I. Bas du rapport

1. Ce rapport a été rédigé sur la base des éléments ci-après (*les feuilles de remplacement qui ont été remises à l'office récepteur en réponse à une invitation faite conformément à l'article 14 sont considérées, dans le présent rapport, comme "initialement déposées" et ne sont pas jointes en annexe au rapport puisqu'elles ne contiennent pas de modifications.*) :

Description, pages:

1-27   version initiale

Revendications, N°:

1-29   version initiale

Dessins, feuilles:

1/3-3/3   version initiale

2. Les modifications ont entrainé l'annulation :

☐   de la description,   pages :

☐   des revendications,   n°ˢ :

☐   des dessins,   feuilles :

3. ☐   Le présent rapport a été formulé abstraction faite (de certaines) des modifications, qui ont été considérées comme allant au-delà de l'exposé de l'invention tel qu'il a été déposé, comme il est indiqué ci-après (règle 70.2(c)) :

4. Observations complémentaires, le cas échéant :

III. Absence de formulation d'opinion quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle

La question de savoir si l'objet de l'invention revendiquée semble être nouveau, impliquer une activité inventive (ne pas être évident) ou être susceptible d'application industrielle n'a pas été examinée pour ce qui concerne :

☐   l'ensemble de la demande internationale.

☒   les revendications n°ˢ 7, 8 (partiellement), 9 (partiellement), 15, 16, 17, 18 (partiellement), 19 (partiellement),

20-23, 24 (partiellement), 25 (partiellement), 29.

parce que :

☒ la demande internationale, ou les revendications nᵒˢ 24 (partiellement), 25 en question, se rapportent à l'objet suivant, à l'égard duquel l'administration chargée de l'examen préliminaire international n'est pas tenue effectuer un examen préliminaire international (*préciser*) :

   voir feuille séparée

☐ la description, les revendications ou les dessins (*en indiquer les éléments ci-dessous*), ou les revendications nᵒˢ en question ne sont pas clairs, de sorte qu'il n'est pas possible de formuler une opinion valable (*préciser*) :

☒ les revendications, ou les revendications nᵒˢ 7, 8 (partiellement), 9 (partiellement), 15, 16, 17, 18 (partiellement), 19 (partiellement), 20-23, 24 (partiellement), 25 (partiellement), 29 en question, ne se fondent pas de façon adéquate sur la description, de sorte qu'il n'est pas possible de formuler une opinion valable.

☐ il n'a pas été établi de rapport de recherche internationale pour les revendications nᵒˢ en question.

IV. Absence d'unité de l'invention

1. En réponse à l'invitation à limiter les revendications ou à payer des taxes additionnelles, le déposant a

   ☐ limité les revendications.

   ☐ payé des taxes additionnelles.

   ☐ payé des taxes additionnelles sous réserve.

   ☐ ni limité les revendications ni payé des taxes additionnelles.

2. ☒ L'administration chargée de l'examen préliminaire international estime qu'il n'est pas satisfait à l'exigence d'unité d'invention et décide, conformément à la règle 68.1, de ne pas inviter le déposant à limiter les revendications ou à payer des taxes additionnelles.

3. L'administration chargée de l'examen préliminaire international estime que, aux termes des règles 13.1, 13.2 et 13.3,

   ☐ il est satisfait à l'exigence d'unité de l'invention.

   ☒ il n'est pas satisfait à l'exigence d'unité de l'invention, et ce pour les raisons suivantes :

   voir f uille séparé

4. En conséquence, les parties suivantes de la demande internationale ont fait l'objet d'un examen préliminaire international lors de la formulation du présent rapport :
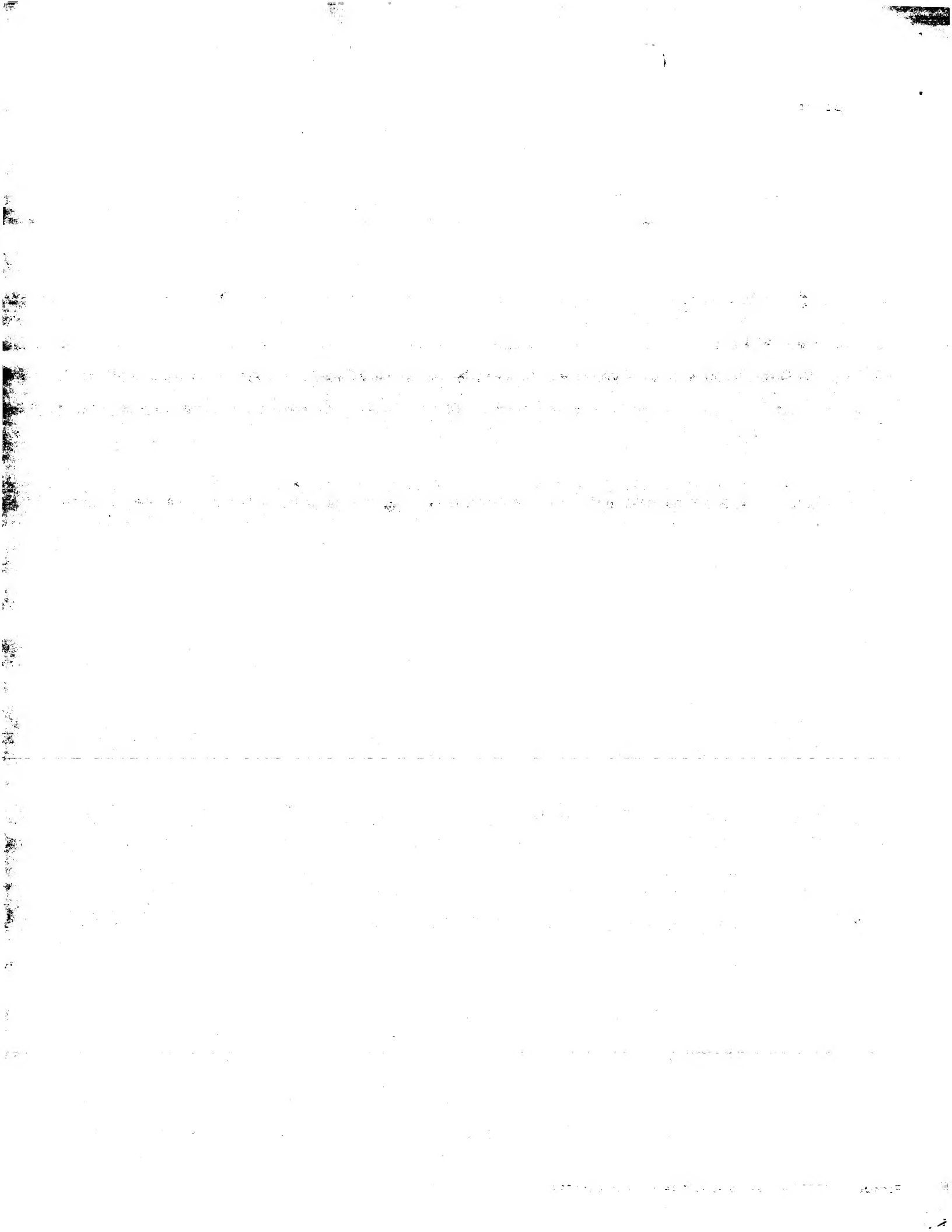
☒ toutes les parties de la demande.

☐ les parties relatives aux revendications n^{os}.

V. Déclaration motivée selon l'article 35(2) quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle; citations et explications à l'appui de cette déclaration

1. Déclaration

Nouveauté	Oui :	Revendications	4-6, 8 (partiellement), 9 (partiellement),10, 11, 12, 14, 24 (partiellement), 25 (partiellement), 26
	Non :	Revendications	1-3, 13, 18 (partiellement), 19 (partiellement), 27, 28
Activité inventive	Oui :	Revendications	
	Non :	Revendications	1-6, 8 (partieellement), 9 (partiellement), 10-14, 18 (partiellement),19 (partiellement), 24 (partiellement), 25 (partiellement), 26, 27, 28
Possibilité d'application industrielle	Oui :	Revendications	1-23, 26-29
	Non :	Revendications	

2. Citations et explications

   voir feuille séparée

VIII. Observations relatives à la demande internationale

Les observations suivantes sont faites au sujet de la clarté des revendications, de la description et des dessins et de la question de savoir si les revendications se fondent entièrement sur la description :

   voir feuille séparée

Concernant le point III

Absence de formulation d'opinion quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle

1. La présente administration considère que l'objet des revendications 7, 8 (partiellement), 9 (partiellement), 15, 16, 17, 18 (partiellement), 19 (partiellement), 24 (partiellement), 25 (partiellement), 29 n'est pas défini en termes techniques et donc ne définit pas clairement l'étendue de la protection demandée (voir objection point VIII paragraphe 2 ci-dessous).
Cette objection concerne les termes et formulations suivants:
- 'composé non peptidique ou non peptidique' (revendications 7, 8 et 9);
- 'ligand' (revendications 15, 18, 19, 24, 25), 'agoniste/antagoniste' (revendications 16, 29), 'inhibiteur' (revendications 16, 17)
Cette objection concerne également l'objet des revendications 20-23 qui est défini par le résultat recherché ('destinée à/au...') et non en termes techniques concrets. En conséquence il ne sera pas émis d'opinion quant à la nouveauté et l'activité inventive de ces revendications.

2. L'IPEA considère également que l'objet des revendications 24 (partiellement) et 25 est visé par les dispositions de la règle 67.1 (iv) PCT. C'est pourquoi il ne sera pas émis d'opinion quant à la question de savoir si l'objet de ces revendications est susceptible d'application industrielle (Article 34(4) a) i) PCT).

Concernant le point IV

Absence d'unité de l'invention

La présente demande n'est pas unitaire car le concept qui relie les différentes revendications, 'polypeptide possédant une activité de type β-sécrétase caractérisé en ce qu'il est capable de cliver de manière spécifique le précurseur naturel du peptide β-amyloïde (APP)', n'est pas nouveau (voir objection point V, paragraphe 1 ci-dessous). En conséquence, la demande se divise en une pluralité d'inventions qui ne sont plus liées entre elles par un concept commun (en fait chaque revendication indépendante de produit et de procédé constitue un groupe d'inventions). Malgré cette objection, le demandeur n'est pas invité à payer des taxes additionnelles. Toutefois cette objection sera suivie par l'IPEA dans le cas où l'objet des revendications de polypeptides ne

serait pas rendu nouveau en modifiant la revendication 1 (comme proposé au point V paragraphe 1 ci-dessous) et dans le cas où le dossier serait poursuivi en phase régionale devant l'OEB.

Concernant le point V

Déclaration motivée selon la règle 66.2(a)(ii) quant à la nouveauté, l'activité inventive et la possibilité d'application industrielle; citations et explications à l'appui de cette déclaration

DOCUMENTS CITES

Il est fait référence aux documents suivants:

> D1: EVIN G ET AL: 'ALZHEIMER'S DISEASE AMYLOID PRECURSOR PROTEIN (ABETAPP): PROTEOLYTIC PROCESSING, SECRETASES AND BETAA4 AMYLOID PRODUCTION' AMYLOID, vol. 1, no. 4, 1994, pages 263-280, XP000602976
>
> D2: EP-A-0 576 152 (LILLY CO ELI) 29 décembre 1993 (1993-12-29)
>
> D3: WO 91 13904 A (CEPHALON INC) 19 septembre 1991 (1991-09-19)
>
> D4: WO 96 40885 A (ATHENA NEUROSCIENCES INC ;CHRYSLER SUSANNA M S (US); SINHA SUKANTO) 19 décembre 1996 (1996-12-19)

NOUVEAUTE ET ACTIVITE INVENTIVE - ARTICLES 33(2) ET (3) PCT

1. Les documents de l'art antérieur décrivent divers polypeptides ayant les mêmes caractéristiques que le polypeptide des revendications 1-3 (D1: page 10 Table 5 et paragraphe 'Searching for the β-secretase'; D2: page 2 lignes 20-22; D3: abstract et page 35 lignes 23-27; D4: Abstract lignes 1-2 et revendications 1et 2). Ces documents décrivent également des anticorps dirigés contre ces polypeptides (D2: page 7 ligne 45; D3: page 54 point b; D4: abstract et revendication 9) ainsi que l'utilisation de ces polypeptides pour la mise en évidence de ligands et/ou d'inhibiteurs (D2: revendication 9; D3: example 8 page 48-50; D4: résumé et pages 22-24 'Screening Assays'). L'objet des <u>revendications 1-3 et 13, 18 (partiellement), 19 (partiellement), 27 et 28</u> n'est donc pas nouveau. Afin de rendre ces revendications nouvelles toutes les caractéristiques des

revendications dépendantes 4 **et** 6 doivent être combinées à celles de la revendication 1.

2. L'objet des revendications de procédé et d'utilisation des revendications 10, 11, 12, 14, 24 (partiellement), 25 (partiellement), 26) de la présente demande n'est pas décrit dans l'état de la technique mis à la disposition de l'examinateur. Par conséquent, ces revendications sont conformes au critère de nouveauté défini par l'Article 33(2) PCT. Cependant étant donné que l'art antérieur décrit la fonction des différents polypeptides ainsi que leur possible utilisation, l'IPEA considère que l'objet de ces revendications n'implique pas d'activité inventive telle que définie par l'Article 33(3) PCT.
Toutefois, dans le cas où le Demandeur modifierait la revendication 1 comme proposé au point 1, l'objet des revendications de procédé et d'utilisation serait alors considéré comme nouveau et inventif.

APPLICATION INDUSTRIELLE - ARTICLES 33(1) ET (4) PCT

Il n'existe pas de critère unifié dans les Etats parties au PCT pour déterminer si les revendications 24 (partiellement) et 25 sont susceptibles d'application industrielle. La brevetabilité peut aussi dépendre de la manière dont les revendications ont été formulées. Ainsi, l'Office européen des brevets ne considère pas comme susceptible d'application industrielle l'objet de revendications d'utilisation d'un composé à des fins médicales. Par contre, peuvent être acceptées des revendications relatives à un composé connu, pour une première utilisation à des fins médicales ainsi que des revendications relatives à l'utilisation d'un tel composé dans la fabrication d'un médicament en vue d'un nouveau traitement médical.

Concernant le point VIII
Observations relatives à la demande internationale

1. L'objet des revendications 3, 7 et 15 est défini par le procédé d'obtention du produit. Le fait que le polypeptide soit obtenu par purification à partir de cellules humaines de sujet non atteint par la maladie d'Alzeimer (revendication 3), que le composé non peptidique ou non exclusivement peptidique soit obtenu par reproduction des motifs actifs.... (revendication 7), que le ligand soit obtenu selon

le procédé de la revendication 14 (revendication 15) ne rend pas ces polypeptides, composés et ligands différents de polypeptides, composés et ligands obtenus par une autre méthode (par exemple par synthèse chimique). Les revendications de produits dans lesquelles les produits sont définis par leur procédé de fabrication (dites **revendications de produits caractérisés par leur procédé d'obtention":"product-by-process" claims**) ne peuvent être acceptées que si les produits en tant que tels satisfont aux conditions requises pour la brevetabilité, et que si la demande ne contient aucune autre information permettant au demandeur de définir le produit de manière satisfaisante par référence à sa composition, à sa structure ou à autre paramètre pouvant être testé (Article 6 PCT).

2.   Les termes 'Agoniste', 'Antagoniste', 'Ligand', 'Inhibiteurs' ne définissent pas un composé par ses caractéristiques techniques mais au moyen de **caractéristiques de fonctionnement**. Ce type de définition ne fait pas référence à un composé tangible ou un groupe de composés, mais à un nombre infini de composés possibles pouvant présentés des compositions chimiques très diverses. En conséquence, la formulation <u>des revendications 7, 8 (partiellement), 9 (partiellement), 15, 16, 17, 18 (partiellement), 19 (partiellement), 24 (partiellement), 25 (partiellement) et 29</u> ne définit pas clairement l'objet pour lequel une protection est demandée (Article 6 PCT).

TRAITE DE C   PERATION EN MATIERE DE B   ETS

PCT

RAPPORT DE RECHERCHE INTERNATIONALE

(article 18 et règles 43 et 44 du PCT)

Référence du dossier du déposant ou du mandataire ST 98014	**POUR SUITE** **A DONNER**	voir la notification de transmission du rapport de recherche internationale (formulaire PCT/ISA/220) et, le cas échéant, le point 5 ci-après
Demande internationale n° PCT/FR 99/01326	Date du dépôt international*(jour/mois/année)* 04/06/1999	(Date de priorité (la plus ancienne) *(jour/mois/année)* 05/06/1998

Déposant

RHONE-POULENC RORER S.A. et al.

Le présent rapport de recherche internationale, établi par l'administration chargée de la recherche internationale, est transmis au déposant conformément à l'article 18. Une copie en est transmise au Bureau international.

Ce rapport de recherche internationale comprend _____4_____ feuilles.

[X] Il est aussi accompagné d'une copie de chaque document relatif à l'état de la technique qui y est cité.

1. **Base du rapport**

   a. En ce qui concerne la **langue**, la recherche internationale a été effectuée sur la base de la demande internationale dans la langue dans laquelle elle a été déposée, sauf indication contraire donnée sous le même point.

   [ ] la recherche internationale a été effectuée sur la base d'une traduction de la demande internationale remise à l'administration.

   b. En ce qui concerne **les séquences de nucléotides ou d'acides aminés** divulguées dans la demande internationale (le cas échéant), la recherche internationale a été effectuée sur la base du listage des séquences :

   [ ] contenu dans la demande internationale, sous forme écrite.

   [ ] déposée avec la demande internationale, sous forme déchiffrable par ordinateur.

   [ ] remis ultérieurement à l'administration, sous forme écrite.

   [ ] remis ultérieurement à l'administration, sous forme déchiffrable par ordinateur.

   [ ] La déclaration, selon laquelle le listage des séquences présenté par écrit et fourni ultérieurement ne vas pas au-delà de la divulgation faite dans la demande telle que déposée, a été fournie.

   [ ] La déclaration, selon laquelle les informations enregistrées sous forme déchiffrable par ordinateur sont identiques à celles du listage des séquences présenté par écrit, a été fournie.

2. [X] **Il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche** (voir le cadre I).

3. [ ] **Il y a absence d'unité de l'invention** (voir le cadre II).

4. En ce qui concerne le **titre**,

   [ ] le texte est approuvé tel qu'il a été remis par le déposant.

   [X] Le texte a été établi par l'administration et a la teneur suivante:

   POLYPEPTIDES POSSEDANT UNE ACTIVITE DE TYPE BETA-SECRETASE

5. En ce qui concerne **l'abrégé**,

   [X] le texte est approuvé tel qu'il a été remis par le déposant

   [ ] le texte (reproduit dans le cadre III) a été établi par l'administration conformément à la règle 38.2b). Le déposant peut présenter des observations à l'administration dans un délai d'un mois à compter de la date d'expédition du présent rapport de recherche internationale.

6. La figure **des dessins** à publier avec l'abrégé est la Figure n° _____

   [ ] suggérée par le déposant.

   [ ] parce que le déposant n'a pas suggéré de figure.

   [ ] parce que cette figure caractérise mieux l'invention.

   [X] Aucune des figures n'est à publier.

RAPPORT DE RECHERCHE INTERNATIONALE

Demande internationale n°

PCT/FR 99/01326

Cadre I Observations - lorsqu'il a été estimé que certaines revendications ne pouvaient pas faire l'objet d'une recherche (suite du point 1 de la première feuille)

Conformément à l'article 17.2)a), certaines revendications n'ont pas fait l'objet d'une recherche pour les motifs suivants:

1. [X] Les revendications nᵒˢ
se rapportent à un objet à l'égard duquel l'administration n'est pas tenue de procéder à la recherche, à savoir:

> Remarque: Bien que les revendications 24 et 25 (partiellement)
> concernent une méthode de traitement du corps humain/animal,
> la recherche a été effectuée et basée sur les effets imputés
> au produit/à la composition.

2. [ ] Les revendications nᵒˢ
se rapportent à des parties de la demande internationale qui ne remplissent pas suffisamment les conditions prescrites pour qu'une recherche significative puisse être effectuée, en particulier:

3. [ ] Les revendications nᵒˢ
sont des revendications dépendantes et ne sont pas rédigées conformément aux dispositions de la deuxième et de la troisième phrases de la règle 6.4.a).

Cadre II Observations - lorsqu'il y a absence d'unité de l'invention (suite du point 2 de la première feuille)

L'administration chargée de la recherche internationale a trouvé plusieurs inventions dans la demande internationale, à savoir:

1. [ ] Comme toutes les taxes additionnelles ont été payées dans les délais par le déposant, le présent rapport de recherche internationale porte sur toutes les revendications pouvant faire l'objet d'une recherche.

2. [ ] Comme toutes les recherches portant sur les revendications qui s'y prêtaient ont pu être effectuées sans effort particulier justifiant une taxe additionnelle, l'administration n'a sollicité le paiement d'aucune taxe de cette nature.

3. [ ] Comme une partie seulement des taxes additionnelles demandées a été payée dans les délais par le déposant, le présent rapport de recherche internationale ne porte que sur les revendications pour lesquelles les taxes ont été payées, à savoir les revendications nᵒˢ

4. [ ] Aucune taxe additionnelle demandée n'a été payée dans les délais par le déposant. En conséquence, le présent rapport de recherche internationale ne porte que sur l'invention mentionnée en premier lieu dans les revendications; elle est couverte par les revendications nᵒˢ

Remarque quant à la réserve

[ ] Les taxes additionnelles étaient accompagnées d'une réserve de la part du déposant.

[ ] Le paiement des taxes additionnelles n'était assorti d'aucune réserve.

Formulaire PCT/ISA/210 (suite de la première feuille (1)) (Juillet 1998)

RAPPORT DE RECHERCHE INTERNATIONALE

A. CLASSEMENT DE L'OBJET DE LA DEMANDE
CIB 6   C12N15/12   C12N15/57   C12N9/64   C07K16/40   C12Q1/37
         A61K39/395   A61K38/48   A61K31/70

Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB

B. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE
Documentation minimale consultée (système de classification suivi des symboles de classement)
CIB 6   C12N   C07K   C12Q   A61K

Documentation consultée autre que la documentation minimale dans la mesure où ces documents relèvent des domaines sur lesquels a porté la recherche

Base de données électronique consultée au cours de la recherche internationale (nom de la base de données, et si réalisable, termes de recherche utilisés)

C. DOCUMENTS CONSIDERES COMME PERTINENTS

Catégorie°	Identification des documents cités, avec, le cas échéant, l'indication des passages pertinents	no. des revendications visées
X	WO 96 40885 A (ATHENA NEUROSCIENCES INC ;CHRYSLER SUSANNA M S (US); SINHA SUKANTO) 19 décembre 1996 (1996-12-19) le document en entier ---	1-3, 7-10, 13-29
X	WO 92 03542 A (UNIV BOSTON) 5 mars 1992 (1992-03-05) le document en entier ---	1,2,13
X	WO 92 07068 A (ATHENA NEUROSCIENCES INC ;LILLY CO ELI (US)) 30 avril 1992 (1992-04-30) le document en entier --- -/--	1-3, 7-10, 13-29

[X] Voir la suite du cadre C pour la fin de la liste des documents   [X] Les documents de familles de brevets sont indiqués en annexe

° Catégories spéciales de documents cités:

"A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent

"E" document antérieur, mais publié à la date de dépôt international ou après cette date

"L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée)

"O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens

"P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée

"T" document ultérieur publié après la date de dépôt international ou la date de priorité et n'appartenenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention

"X" document particulièrement pertinent; l'inven tion revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive par rapport au document considéré isolément

"Y" document particulièrement pertinent; l'inven tion revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier

"&" document qui fait partie de la même famille de brevets

Date à laquelle la recherche internationale a été effectivement achevée	Date d'expédition du présent rapport de recherche internationale
2 septembre 1999	08/09/1999

Nom et adresse postale de l'administration chargée de la recherche internationale	Fonctionnaire autorisé
Office Européen des Brevets, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

Formulaire PCT/ISA/210 (deuxième feuille) (juillet 1992)

C.(suite) DOCUMENTS CONSIDERES COMME PERTINENTS		
Catégorie°	Identification des documents cités, avec,le cas échéant, l'indicationdes passages pertinents	no. des revendications visées
X	NELSON R B ET AL: "CLIPSIN, A CHYMOTRYPSIN-LIKE PROTEASE IN RAT BRAIN WHICH IS IRREVERSIBLY INHIBITED BY ALPHA-1-ANTICHYMOTRYPSIN" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 265, no. 7, 5 mars 1990 (1990-03-05), pages 3836-3843, XP002011909 le document en entier ---	1,2,13
X	EP 0 576 152 A (LILLY CO ELI) 29 décembre 1993 (1993-12-29) le document en entier ---	1-3,7-9, 13-16, 24-29
A	EVIN G ET AL: "ALZHEIMER'S DISEASE AMYLOID PRECURSOR PROTEIN (ABETAPP): PROTEOLYTIC PROCESSING, SECRETASES AND BETAA4 AMYLOID PRODUCTION" AMYLOID, vol. 1, no. 4, 1994, pages 263-280, XP000602976 ---	
X	WO 91 13904 A (CEPHALON INC) 19 septembre 1991 (1991-09-19) page 35 - page 63 ---	1-3,13
X	EP 0 569 777 A (MILES INC) 18 novembre 1993 (1993-11-18) revendications; exemple 10 ---	1-3, 7-10, 13-29
X	BROWN A M ET AL: "EVALUATION OF CATHEPSINS D AND G AND EC 3.4.24.15 AS CANDIDATE BETA-SECRETASE PROTEASES USING PEPTIDE AND AMYLOID PRECURSOR PROTEIN SUBSTATES" JOURNAL OF NEUROCHEMISTRY, vol. 66, no. 6, 1996, pages 2436-2445, XP000602767 le document en entier -----	1-3

1

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9640885	A	19-12-1996	US	5762805	A	09-06-1998
			US	5744346	A	28-04-1998
			AU	6383396	A	30-12-1996
			EP	0871720	A	21-10-1998
WO 9203542	A	05-03-1992	AU	643835	B	25-11-1993
			AU	8538791	A	17-03-1992
			CA	2088776	A	18-02-1992
			EP	0546084	A	16-06-1993
			HU	69771	A	28-09-1995
			JP	6503948	T	12-05-1994
			US	5849560	A	15-12-1998
			US	5200339	A	06-04-1993
WO 9207068	A	30-04-1992	US	5292652	A	08-03-1994
			US	5424205	A	13-06-1994
EP 0576152	A	29-12-1993	AU	3986493	A	02-12-1993
			BR	9302075	A	30-11-1993
			CA	2096911	A	29-11-1993
			CZ	9300982	A	16-02-1994
			FI	932425	A	29-11-1993
			HU	69612	A	28-09-1995
			JP	6062855	A	08-03-1994
			MX	9303082	A	30-06-1994
			NO	931889	A	29-11-1993
			PL	299053	A	21-02-1994
			US	5733768	A	31-03-1998
WO 9113904	A	19-09-1991	AU	661270	B	20-07-1995
			AU	7465491	A	10-10-1991
			CA	2077665	A	06-09-1991
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			EP	0732399	A	18-09-1996
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Clipsin, a Chymotrypsin-like Protease in Rat Brain Which Is Irreversibly Inhibited by α-1-Antichymotrypsin*

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The protease inhibitor α-1-antichymotrypsin, which binds to chymotrypsin-like enzymes in a sodium dodecyl sulfate-resistant manner, has been shown recently to be both a normal constituent of brain and an integral component of the neuritic plaques that form in Down's syndrome and Alzheimer's disease. We have now identified in rat brain a M_r 25,000 α-1-antichymotrypsin-binding protein classified as a chymotrypsin-like protease by its inhibitor profile and substrate specificity. Release of ^{125}I-labeled breakdown products from bands containing the protease in substrate-linked polyacrylamide gels was examined in parallel with hydrolysis of tetrapeptide chromogenic substrates in vitro to establish conditions under which the M_r 25,000 protease was the only activity being measured in vitro. The protease was completely membrane associated but was extractable using 1 M MgCl₂; prior extraction of detergent- and low ionic strength-soluble proteins from membranes was used to increase its specific activity. The formation of sodium dodecyl sulfate-resistant bonds between human α-1-antichymotrypsin and the protease ($k_{assoc} = 2.9 \times 10^6$ M^{-1} s^{-1}) was used to titrate the concentration of free protease solubilized from membranes. The protease cleaved both succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and methoxy-succinyl-Ala-Ala-Pro-Met-p-nitroanilide, the latter being of interest because cleavage after a methionine residue is predicted to generate the amino terminus of the neuritic plaque component β-amyloid from its precursor protein. In fact, the solubilized protease degraded 90% of membrane-associated β-amyloid precursor protein detected by Western blot analysis. The protease was kinetically distinct from both chymotrypsin and cathepsin G in direct comparisons and did not match kinetic values published for the rat mast cell proteases against comparable substrates; we therefore refer to the protease with the descriptive acronym clipsin (for chymotrypsin-like protease). Proteases similar to and potentially identical to clipsin were detected by enzymography in other organs from rat (most notably spleen and adult lung). The enzyme in brain was distinguished by a narrow window of elevated activity surrounding postnatal day 5, which was 12–14-fold higher than levels in day 1 or adult brain. Because independent lines of evidence suggest that a brain chymotrypsin-like protease may be involved in the etiology of Down's syndrome and Alzheimer's disease, clipsin is discussed as a candidate for such a role.

A growing body of evidence suggests that protease/protease inhibitor systems play a diversity of roles in central nervous system functions. The Ca^{2+}-dependent cysteine proteases (calpains I and II) have been linked to long term synaptic modification (Lynch and Baudry, 1984; Siman et al., 1987) and, in our laboratory, to neurotoxicity induced by excitatory amino acids (Siman and Noszek, 1988; Siman et al., 1989b). An inhibitor of serine proteases has been identified as a neurite-promoting factor (Gloor et al., 1986); moreover, the serine protease thrombin was shown to be responsible for inhibition of neurite outgrowth (Gurwitz and Cunningham, 1988). We have found recently that thrombin and thrombin inhibitors can reversibly regulate the differentiation state of cultured primary glial cells.[1] We have also described recently a family of Ca^{2+}-dependent metalloproteases in brain which are released from neurons and which, by analogy to their role in other tissues, may allow developing neurites to penetrate the extracellular matrix (Nelson and Siman, 1989).

Recent evidence suggests that protease systems may also participate in certain neuropathologies. In both Down's syndrome and Alzheimer's disease, an insoluble peptide termed β-amyloid aggregates into filamentous deposits known as neuritic plaques (Allsop et al., 1983; Glenner and Wong, 1984; Masters et al., 1985; Castano et al., 1986; Kirschner et al., 1987). β-Amyloid is derived from one or more β-amyloid precursor proteins (β-APP[2]; Kang et al., 1987; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988), which are normal neuronal constituents (Card et al., 1988). The generation of β-amyloid is postulated to result from inappropriate processing of β-APP by one or more proteases (Glenner and Wong, 1987; Kang et al., 1987; Grundke-Iqbal et al., 1989; Weidemann et al., 1989). Specifically, the amino terminus of β-amyloid is formed through hydrolysis of β-APP on the carboxyl side of methionine^{596} (Kang et al., 1987). Based upon the affinities of known proteases for primary protein structure, such a cleavage event is predicted to result through the

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[1] R. B. Nelson and R. Siman, submitted for publication.
[2] The abbreviations used are: β-APP, β-amyloid precursor protein; α-1-AChy, α-1-antichymotrypsin; boro, (R)-acetamido-2-phenylethane boronic acid, where R is the L-amino acid preceded by the prefix "boro"; Bz, benzyl; CHAPS, 3-[(3-choloamidopropyl)-dimethylammonio]-1-propanesulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]; MeO-, methoxy-; Suc, succinyl; RMCP(s), rat mast cell protease(s).

action of a chymotrypsin-like enzyme (Barrett and Mc-Donald. 1980; Lorand, 1981).

β-Amyloid is one of two identified integral components of neuritic plaques. The second known component is α-1-antichymotrypsin (α-1-AChy; Abraham et al., 1988), a protease inhibitor that forms sodium dodecyl sulfate-resistant bonds with chymotrypsin-like enzymes (Travis et al., 1978). It is not yet known whether α-1-AChy might be associated with neuritic plaques by virtue of its binding to a chymotrypsin-like protease which itself is an integral plaque component. However, given that the two integral plaque components identified to date may have functional links with chymotrypsin-like proteases, the hypothesis that such enzymes are involved in the formation of neuritic plaques is a compelling one.

Because α-1-AChy has also been reported in normal brain (Justice et al., 1987; Abraham et al., 1989), we were encouraged to search for a corresponding chymotrypsin-like protease in normal brain which would provide a starting point for studying chymotrypsin-like activity in normal neural functioning and in degenerative neuropathologies such as Down's syndrome and Alzheimer's disease. In the present paper, we surveyed brain tissue from rats of various ages using a sensitive enzymographic protocol described previously (Nelson and Siman, 1989). We report here the detection and characterization of a chymotrypsin-like protease termed clipsin (for chymotrypsin-like protease) which binds to α-1-AChy in a sodium dodecyl sulfate-resistant manner, preferentially degrades β-APP, and shows high activity for a period of a few days in neonatal rat brain.

EXPERIMENTAL PROCEDURES

Materials—Bovine α-chymotrypsin treated with 1-chloro-3-tosylamido-7-amino-2-heptanone, human leukocyte cathepsin G, MeO-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide, and Suc-Ala-Ala-Met-*p*-nitroanilide were obtained from Sigma. Human α-1-AChy was from Calbiochem. MeO-Suc-Ala-Ala-Pro-boro-Phe-OH, MeO-Suc-Ala-Ala-Pro-boro-Ala-OH, MeO-Suc-Ala-Ala-Pro-boro-Val-OH, and Bz-Pro-Phe-boro-Arg-OH were gifts of Dr. Charles Kettner, DuPont Experimental Station, Wilmington, DE. Antiserum 385, raised against a synthetic peptide corresponding to residues 676–695 of β-APP (numbering according to Kang et al., 1987), was prepared and characterized as described previously (Card et al., 1988; Siman et al., 1989a).

Preparation of Clipsin Extract—All procedures were performed at 4 °C unless otherwise indicated. Forebrains from 5-day-old rats were stripped of meninges and homogenized in 20 volumes of 50 mM Hepes (pH 7.5), 1 mM EDTA, and 1% Triton X-100 (or 1% CHAPS). The homogenate was allowed to remain for 30 min with occasional vortexing and was then centrifuged 1 h at 40,000 rpm (150,000 × g_{avg}) in a Sorvall 50.38 rotor. For the low ionic strength extraction, the pellet was resuspended to original volume in 1 mM Hepes (pH 7.5), 0.1 mM EDTA made up in 18 mega-ohm resistance water that had been passed through a chelating resin column (Chelex 100). The resuspension was shaken in a water bath for 1 h at 37 °C and then centrifuged as above. Pellets were resuspended to 10% original volume in 50 mM Hepes (pH 7.5), 1 M MgCl₂, and 0.1% Brij 35, and allowed to remain on ice for 30 min with occasional vortexing. Samples were centrifuged as above for 1 h. The supernatant from this extraction was dialyzed exhaustively against 20 mM Hepes (pH 7.5), 0.5 M NaCl, 1 mM EDTA, and 0.1% Brij 35. The resulting Mg²⁺ extract could be frozen with no appreciable loss of activity and was stored at −80 °C for subsequent use in the *in vitro* assay described below.

For the ontogeny and tissue distribution studies, in which Mg²⁺ extracts were prepared on a small scale from multiple sources, the procedure was identical to that above except that centrifugation was carried out in a tabletop ultracentrifuge using a TLA-100.2 Beckman rotor. Each centrifuge run was for 20 min at 80,000 rpm (350,000 × g_{max}). For the tissue distribution study, both the 5-day-old rats and adult rats were deeply anesthetized with Pseudochlor (1 mg/kg) prior to being perfused intracardially with ice-cold phosphate-buffered saline. This removed blood cells and serum as contributors to the protease complement detected in the various organs. Following the

perfusion. tissues were dissected out on ice, minced, sonicated in the detergent-containing buffer (see above), and processed for Mg²⁺ extract preparation. Final protein concentration of each Mg²⁺ extract was adjusted using the method of Bradford (1976).

Enzymography—Protease activity was assayed in ¹²⁵I-gelatin-containing polyacrylamide gels as described previously (Nelson and Siman, 1989). Following removal of sodium dodecyl sulfate from the gels and incubation of the gels in conditions favoring reactivation of protease-containing bands, gelatinolytic activity is detected through electrophoretic transfer of ¹²⁵I-labeled breakdown products onto nitrocellulose paper, followed by autoradiography. A variety of proteases has been shown to retain activity using this type of assay (Heussen and Dowdle, 1980; Miskin and Soreq, 1981; Nelson and Siman, 1989). The effects of inhibitors on electrophoretically separated proteases were tested by preequilibrating the gelatin-containing polyacrylamide gel strips in inhibitor-containing buffers for 20 min at 4 °C prior to activating proteases by incubation of the gels in a humidified chamber at 37 °C.

Assays with Chromogenic Peptide Substrates—The hydrolysis rates of several tetrapeptide *p*-nitroanilides by clipsin and other chymotrypsin-like proteases were measured by incubating 180 μl of enzyme in Mg²⁺ extract buffer with 20 μl of *p*-nitroanilide substrate in dimethyl sulfoxide. Reactions were usually carried out for 60 min and then stopped by the addition of 100 μl of 200 μg/ml soybean trypsin inhibitor. In pilot studies, we determined that the chymotrypsin-like enzymes, including clipsin, were stable at 50 °C as reported previously (Barrett, 1981); therefore, kinetic studies were run at this temperature to increase the reaction rate. Using Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate, we also adjusted the concentrations of chymotrypsin and cathepsin G to obtain reaction rates approximately equal to that of a 5-fold dilution of Mg²⁺ extract. Under these conditions, substrate hydrolysis was linear for at least 90 min. The increase in absorbance at 405 nm was measured using an Artek microtiter plate reader and converted to concentration of nitroaniline generated using an ε of 1.07×10^4 M⁻¹ cm⁻¹ (Virca et al., 1984). The kinetic constants were determined from initial rates of hydrolysis by the Lineweaver-Burk method and are based on triplicate determinations at five or six separate substrate concentrations chosen so that values of reciprocal concentrations would be evenly proportioned. Correlation coefficients were greater than 0.99.

In cases in which boronic acid peptide inhibitors or α-1-AChy were tested *in vitro*, 160 μl of enzyme in Mg²⁺ extract buffer was preincubated with 20 μl of inhibitor in Mg²⁺ extract buffer for 20 min at 37 °C before addition of 20 μl of substrate solution in dimethyl sulfoxide for 1 h and termination with 100 μl of 200 μg/ml soybean trypsin inhibitor.

Titration with α-1-AChy—α-1-AChy produces a progressive time-dependent inhibition due to its formation of sodium dodecyl sulfate-resistant complexes with its substrates (Travis et al., 1978). After determining that concentrations of α-1-AChy ~3.5 nM or higher produced rapid inhibition of clipsin activity in 20% Mg²⁺ extract, we explored the time course of complete association using lower α-1-AChy concentrations. 20% Mg²⁺ extract was incubated with a range of α-1-AChy concentrations in Mg²⁺ extract buffer at 37 °C. After fixed intervals, 180-μl aliquots were removed and mixed with 20 μl of substrate solution (2 mM final) and then incubated at 50 °C for 1 h before terminating the reaction with 100 μl of 200 μg/ml soybean trypsin inhibitor. Initial velocities in the presence of inhibitor were compared with the control (no α-1-AChy), and percent activities were calculated. At all time points, linear inhibition curves were obtained allowing the determination of a titration point for clipsin using the inhibitor (Beatty et al., 1980; Kettner and Shenvi, 1984). We then graphed these titration points with respect to time to determine when α-1-AChy inhibition of clipsin approached completion. The asymptotic titration value was provisionally used as the titrated concentration of clipsin in Mg²⁺ extract. Verification that this binding was rapid and irreversible was through determination of the association rate constant (see below) and through the demonstration on enzymographic gels that this binding was stable in the presence of sodium dodecyl sulfate.

Determination of Association Rate Constant—The association rate constant for α-1-AChy binding to clipsin was determined under second-order conditions as described previously for α-1-AChy binding to other chymotrypsin-like proteases (Beatty et al., 1980). Using the titrated concentration of clipsin, an equimolar concentration of α-1-AChy was preincubated with Mg²⁺ extract for various periods of time before addition of 2 mM substrate which stopped the reaction and was used to measure residual enzyme activity. The half-life time of

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reaction (t_a) was calculated from a plot of t^{-1} versus t, and in turn used to calculate the association rate constant k_{assoc} through the following $k_{assoc} = 1/t_a \cdot [E_T]$, where $[E_T]$ stands for the total enzyme concentration (Beatty et al., 1980).

Endogenous Substrates Assay—Mg²⁺ extract at 60% (~4.2 nM clipsin final) was incubated with a crude membrane fraction from adult rat cortex (2.8 mg/ml protein final; Siman et al., 1989b). Proteolysis reactions were performed at 37 °C for 1 h before quenching the reactions with sodium dodecyl sulfate-containing electrophoresis sample buffer and heating the samples to 90 °C for 5 min. Overall breakdown of proteins was determined by Coomassie Blue staining of gels, whereas β-APP degradation was assessed by Western blotting of identical gels. β-APP was visualized as 110–130-kDa bands using antiserum 385 as described previously (Siman et al., 1989a). β-APP-immunostained polypeptides on Western blots and the corresponding major Coomassie Blue-stained protein bands on sodium dodecyl sulfate-containing polyacrylamide gels were quantified by scanning laser densitometry.

RESULTS

Subcellular Fractionation and Solubilization of Clipsin—In pilot studies, we identified clipsin using enzymographic gels as a M_r 25,000 proteolytic activity present in 1-week-old but not adult rat brain; consequently, we used the former source in further characterization of the protease. Clipsin was detected only in particulate fractions of rat brain after ultracentrifugation, and it proved to be completely resistant to solubilization by treatment of membranes with detergents (1% Triton X-100 or 1% CHAPS) or a low ionic strength buffer (Fig. 1A). These treatments were therefore used to increase the specific activity of the protease and to remove other activities. The gelatinolytic activity of clipsin detected by enzymography increased with successive membrane treat-

ments. Because gelatin—a general protease substrate used in this type of assay—is unlikely to be a preferred clipsin substrate, this observation most likely indicates that the polyacrylamide-bound ¹²⁵I-labeled gelatin competes as substrates for clipsin. The use of enzymography makes removal of endogenous inhibitors an unlikely explanation for the increasing activity because: (a) proteases are electrophoretically separated from endogenous inhibitors prior to being assayed if such interactions are disrupted by sodium dodecyl sulfate; and (b) inhibitor interactions that are not disrupted by sodium dodecyl sulfate would not be expected to reverse with further fractionation steps.

In the presence of 5 mM Ca²⁺, clipsin activity was partially inhibited, and a number of higher molecular weight protease activities were revealed (Fig. 1B). These activities, which represent a family of Ca²⁺-dependent brain metalloproteases we described previously (Nelson and Siman, 1989), were largely removed by the membrane extraction steps. The relative enrichment of clipsin activity versus total protein is illustrated by Coomassie Blue protein stains (Fig. 1C) of the various protease-containing fractions from Fig. 1, A and B. Although the enzymographic technique is only linear over a restricted range, it appears that greater than 50% of clipsin can be solubilized by a 30-min treatment of the low ionic strength-extracted membrane preparation with 1 M MgCl₂ (Fig. 1D). Attempts to extract a greater percentage of activity by increasing the extraction temperature resulted in an overall decrease in activity. After pelleting out membranes by centrifugation, the supernatant from this Mg²⁺ extraction was dialyzed against Mg²⁺ extract buffer (see "Experimental Procedures") and used for subsequent in vitro experiments.

General Inhibitor Profile of Clipsin—Clipsin was initially characterized using enzymographic gels to distinguish it from any other protease activities potentially present in the Mg²⁺ extract. Because clipsin (left lane of each panel in Fig. 2) showed no dependence on Ca²⁺, and traces of Ca²⁺-dependent metalloprotease activity were detectable in the Mg²⁺ extract
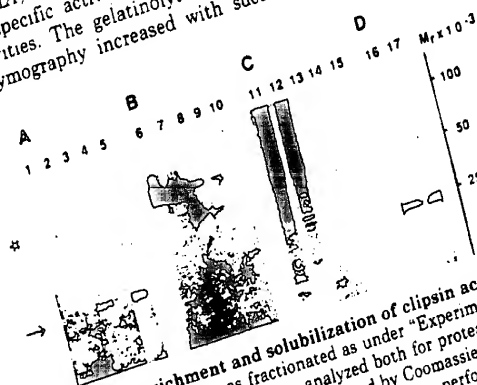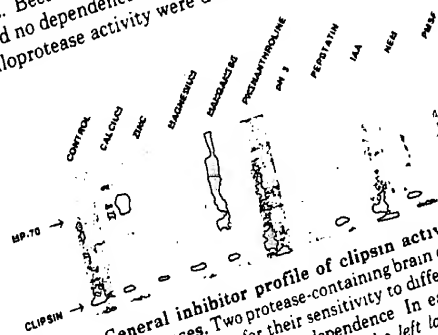

FIG. 1. **Enrichment and solubilization of clipsin activity.** 5-day-old rat forebrain was fractionated as under "Experimental Procedures," and the fractions were analyzed both for protease activity and protein content by enzymography and by Coomassie Blue staining of proteins, respectively. Enzymography was performed as described previously (Nelson and Siman, 1989). Bands on the autoradiographs represent ¹²⁵I-labeled breakdown products released from protease-containing bands in substrate-linked 12.5% polyacrylamide gels, which were then electrophoretically transferred to nitrocellulose. A, enzymograph of protease activities from the fractions indicated below, assayed in the presence of 1 mM EDTA. Arrow denotes clipsin activity. B, enzymograph of protease activities from the fractions indicated below, assayed in the presence of 5 mM Ca²⁺. Asterisk denotes the major metalloprotease activity MP-70. C, Coomassie Blue stain of proteins present in the fractions from A and B. Lanes 1, 6, and 11, Triton X-100-containing homogenate prior to centrifugation; lanes 2, 7, and 12, supernatant from detergent extraction of membranes; lanes 3, 8, and 13, pellet from detergent extraction of membranes resuspended to original volume; lanes 4, 9, and 14, supernatant from low ionic strength extraction of membranes; lanes 5, 10, and 15, pellet from low ionic strength extraction of membranes resuspended to original volume. D, solubilization of clipsin from low ionic strength extracted membranes. Gels were assayed as in A. Lane 16, supernatant from 1 M MgCl₂ extraction of membranes; lane 17, pellet from 1 M MgCl₂ extraction of membranes resuspended to volume of supernatant (0.10 of original volume). Results are representative of three different experiments.

FIG. 2. **General inhibitor profile of clipsin activity versus other brain proteases.** Two protease-containing brain extracts were compared by enzymography for their sensitivity to different protease inhibitors and their divalent cation dependence. In each substrate-containing gel strip (10% polyacrylamide), the left lane was loaded with clipsin-enriched Mg²⁺ extract and the right lane with soluble proteins from embryonic day-16 rat forebrain, a source enriched in metalloprotease activity (Nelson and Siman, 1989). After removal of sodium dodecyl sulfate from the gels, gel strips were separated and preequilibrated in 50 mM Tris-HCl (pH 7.5) plus the inhibitor or divalent cation indicated at the top of each panel. All conditio[n]s (pH 3.0) was used to buffer the low pH condition. All conditio[ns] except those testing effects of divalent cations also included 1 m[M] EDTA. Preequilibration was for 20 min at 4 °C prior to incubat[ion] for 1 h at 37 °C in a humidified chamber to activate prote[ase]. Concentrations used were 1 mM C[a²⁺], mM Zn²⁺, 1 mM Mg²⁺, 1 mM Mn²⁺, 1 mM 1,10-o-phenanthrolin[e], µM pepstatin, 5 mM iodoacetic acid (IAA), 10 mM N-ethylmal[eimide] (N-EM), 1 mM phenylmethylsulfonyl fluoride (PMSF), and diisopropyl fluorophosphate (DFP). Results are representativ[e] experiments.

clipsin activity was routinely assayed in the presence of 1 mM EDTA. No other proteases were detectable in Mg^{2+} extract under these conditions (Fig. 2, *control*). As demonstrated previously, the brain metalloproteases (*right lane* of each panel in Fig. 2) were completely dependent on Ca^{2+} for activity (Nelson and Siman, 1989). Although clipsin showed some inhibition by Ca^{2+} at 5 mM concentrations (Fig. 1), it showed little or no inhibition by several divalent cations, including Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+} at 1 mM concentrations. Lack of complete inhibition by mM Zn^{2+} or by 1,10-*o*-phenanthroline indicated further that clipsin was not one of the brain metalloproteases (Nelson and Siman, 1989). Clipsin was not active at low pH, nor was it inhibited by pepstatin, suggesting that it was not an aspartic protease. Clipsin was also not inhibited by iodoacetic acid or *N*-ethylmaleimide, agents specific for cysteine proteases. Only phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate inhibited clipsin activity, indicating its probable identity as a serine protease. Although these two inhibitors have a broad specificity for serine proteases, they both bind relatively slowly, accounting for the partial activity of clipsin remaining on the gels.

Subclassification of Clipsin as a Chymotrypsin-like Enzyme—A group of synthetic peptide boronic acids was next tested as potential inhibitors of clipsin. These peptide inhibitors, which have been described previously (Kettner and Shenvi, 1984), rapidly inhibit different subtypes of serine proteases depending on the target amino acid at the P1 position of the peptide. We found that clipsin was preferentially inhibited by MeO-Suc-Ala-Ala-Pro-boro-Phe-OH (Fig. 3, *lane 2*), an inhibitor specific for chymotrypsin-like enzymes, but not by MeO-Suc-Ala-Ala-Pro-boro-Ala-OH and MeO-Suc-Ala-Ala-Pro-boro-Val-OH (Fig. 3, *lanes 3* and *4*), inhibitors of leukocyte and pancreatic elastase, respectively, and only marginally by Bz-Pro-Phe-boro-Arg-OH, an inhibitor of trypsin-like serine proteases (Fig. 3, *lane 5*). Based on this characterization, we established an *in vitro* assay of Mg^{2+} extract using Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide, a chromogenic substrate selective for chymotrypsin-like proteases. Mg^{2+} extract contained activity capable of hydrolyzing the

chromogenic substrate, suggesting the presence of an active form of clipsin *in vitro* and indicating low or nonexistent levels of potential endogenous clipsin inhibitors in Mg^{2+} extract (Fig. 3). We therefore measured the ability of the various peptide boronic acids to inhibit Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide hydrolysis by Mg^{2+} extract *in vitro* and compared it with the inhibition of clipsin activity seen on the enzymographs (Fig. 3, ΔOD values). The two methods yielded identical inhibitory profiles, further suggesting that the Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide-hydrolyzing activity in Mg^{2+} extract was due to clipsin as identified on enzymographs.

Binding of α-1-AChy to Clipsin—α-1-AChy is an irreversible inhibitor of chymotrypsin-like proteases (Travis *et al.*, 1978). Moreover, α-1-AChy immunoreactivity and mRNA have been reported in brain (Justice *et al.*, 1987; Abraham *et al.*, 1988; 1989), making α-1-AChy a potential endogenous inhibitor of clipsin. We therefore pursued the characterization of clipsin as a chymotrypsin-like protease by determining
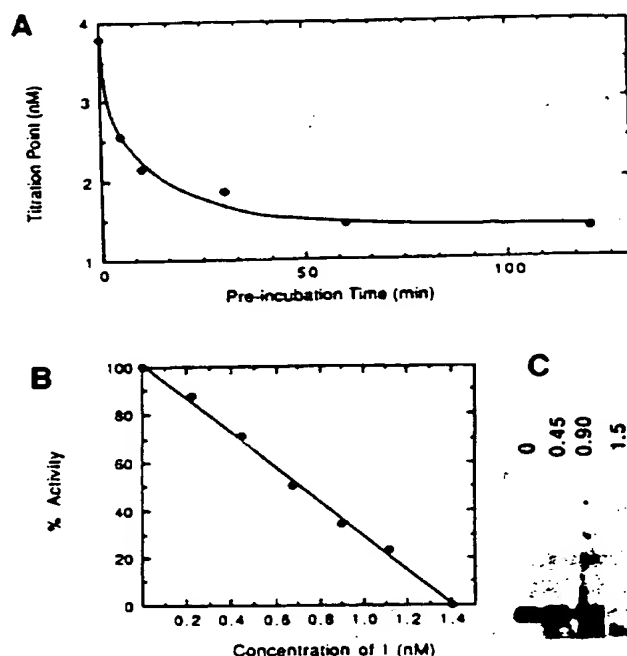
FIG. 4. **Titration of clipsin activity using α-1-AChy.** Mg^{2+} extract was preincubated with various concentrations of α-1-AChy in Mg^{2+} extract buffer at 37 °C. 180-μl aliquots were removed at various intervals and combined with 20 μl of Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide in dimethyl sulfoxide for 1 h at 50 °C. Final concentrations of Mg^{2+} extract and substrate were 20% and 2 mM, respectively. Linear inhibition curves of percent clipsin activity remaining *versus* concentration of α-1-AChy were obtained at each time point measured, and the curves were extrapolated to determine the α-1-AChy concentration necessary to reduce clipsin activity to 0%. *A*, a plot of titration points for clipsin activity *versus* time of preincubation with α-1-AChy. Each titration point on the graph is an average value from two titration curves generated at each time point. Individual points were measured in triplicate. *B*, the titration curve obtained at $t = 120$ min, at which time the titration point had reached its asymptotic value. *I* represents the inhibitor α-1-AChy. The values on this graph are averaged from two separate determinations. *C*, enzymograph of representative samples from which the values in *B* were generated. The gel (10% polyacrylamide) was preincubated in 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA prior to incubation at 37 °C for 4 h to allow protease activity. The extended incubation time, which is past the linear range of this assay, was used to detect lower levels of residual protease activity in samples close to the titration point of clipsin. The nM concentration of α-1-AChy co-incubated with 20% Mg^{2+} extract is indicated above each *lane*. Results are representative of four experiments.

CLIPSIN →

ΔOD: 100 8 97 94 84

FIG. 3. **Effects of peptide boronic acids on clipsin activity measured *in vitro* and by enzymography.** Mg^{2+} extract was separated on substrate-containing gels (10% polyacrylamide) and tested for activity in the presence of various peptide boronic acid serine protease inhibitors (10 μM final concentrations) as in Fig. 2. Parallel samples of Mg^{2+} extract were tested for inhibition of chymotrypsin-like activity against Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide *in vitro*. In the *in vitro* assay, Mg^{2+} extract at 20% final (or 1.4 nM clipsin) was preincubated for 10 min at 37 °C with one of the peptide boronic acids (10 μM final concentration) in Mg^{2+} extract buffer prior to addition of 2 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide for 1 h at 37 °C. The reaction was terminated with 200 μg/ml soybean trypsin inhibitor. The values generated represent change in absorbance (ΔOD) as a percentage of the control value (100%) and are listed at the *bottom* of the corresponding *panel* from the enzymographic analysis. *Lane 1*, control (addition of Mg^{2+} extract buffer alone); *lane 2*, MeO-Suc-Ala-Ala-Pro-boro-Phe-OH; *lane 3*, MeO-Suc-Ala-Ala-Pro-boro-Ala-OH; *lane 4*, MeO-Suc-Ala-Ala-Pro-boro-Val-OH; and *lane 5*, Bz-Pro-Phe-boro-Arg-OH. Enzymographic analysis was performed twice. The *in vitro* values are the average of three determinations.

whether α-1-AChy could irreversibly inhibit clipsin. Pilot experiments determined that α-1-AChy was an effective inhibitor of clipsin activity *in vitro* (using Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate). To determine the time course of this inhibition, we first constructed titration curves of percent residual clipsin activity *versus* α-1-AChy concentration measured after various preincubation times. Each of the plots was linear and yielded an extrapolated titration point of clipsin activity. These titration points are in turn plotted against preincubation time in Fig. 4A. The plot shows that the titration of clipsin with α-1-AChy became asymptotic between 60 and 120 min of preincubation. The titration curve at 120 min yielded a value of 1.4 nM α-1-AChy necessary to inhibit 100% clipsin activity (Fig. 4B). Assuming a 1:1 stoichiometry for α-1-AChy binding to clipsin (based on previously determined α-1-AChy-binding stoichiometries with chymotrypsin and cathepsin G; Travis *et al.*, 1978) and a molecular weight of 25,000 for clipsin, this indicates a 35 ng/ml concentration of clipsin/total Mg^{2+} extract protein concentration of 200 μg/ml. To determine whether clipsin was similar to other chymotrypsin-like enzymes in forming sodium dodecyl sulfate-resistant bonds with α-1-AChy, we used the enzymographic assay to analyze further the samples used to generate Fig. 4B. After separation of proteins on the gelatin-containing polyacrylamide gels, protease activity was allowed to continue to exhaustion (out of the linear range of the assay) to enhance differences between samples close to the titration point of clipsin. Clipsin activity disappeared from the gels between 0.9 and 1.5 nM added α-1-AChy, indicating (a) that α-1-AChy formed complexes with clipsin which survived exposure to sodium dodecyl sulfate; and (b) that the concentration range in which α-1-AChy bound to all free clipsin in the Mg^{2+} extract fraction agreed with the titration point calculated from the *in vitro* assay.

To standardize our estimates of enzyme concentration for kinetic studies, we also used α-1-AChy to titrate the activity in commercial preparations of human cathepsin G and bovine chymotrypsin. Chymotrypsin was titrated at 100-fold higher concentrations of enzyme and inhibitor due to its relatively low rate of association with α-1-AChy (Beatty *et al.*, 1980). On this basis, cathepsin G and chymotrypsin contained 88% and 96%, respectively, of their theoretical activities. Such values are reasonable assuming less than 100% active protease and/or the presence of impurities in the preparations. The kinetic values generated in Table I reflect adjustments in enzyme concentration based on titrated values.

Using the titrated value for clipsin concentration in the Mg^{2+} extract, we combined equimolar concentrations of clipsin and α-1-AChy to determine the association rate constant between these two proteins, as described previously (Equation 4 in Beatty *et al.*, 1980). The k_{assoc} was determined to be 2.9 × 10^6 M^{-1} s^{-1}, a value falling between those determined previously for chymotrypsin and cathepsin G (Beatty *et al.*, 1980).

Kinetics of Substrate Hydrolysis by Clipsin Versus Other Chymotrypsin-like Proteases—We tested the ability of clipsin to hydrolyze several other peptide-nitroanilide substrates, including Suc-Ala-Ala-Pro-Leu-*p*-nitroanilide, MeO-Suc-Ala-Ala-Pro-Val-*p*-nitroanilide, Bz-Arg-*p*-nitroanilide, and MeO-Suc-Ala-Ala-Pro-Met-*p*-nitroanilide. We found detectable hydrolysis only with the last substrate (data not shown), which is also a substrate for chymotrypsin-like proteases (Nakajima *et al.*, 1979). We therefore used both MeO-Suc-Ala-Ala-Pro-Met-*p*-nitroanilide and Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide to do a kinetic comparison of clipsin *versus* bovine chymotrypsin and human leukocyte cathepsin G. As shown in Table I, clipsin could be distinguished kinetically from both cathepsin G and chymotrypsin. Clipsin was similar to cathepsin G in having a greater affinity for the methionine- *versus* the phenylalanine-containing nitroanilide substrate and in having a similar catalytic efficiency to cathepsin G against the methionine-containing substrate. In contrast to cathepsin G, however, clipsin was 11-fold more catalytically efficient against the phenylalanine-containing *p*-nitroanilide substrate. Chymotrypsin kinetic constants were also distinct from clipsin in that chymotrypsin had a lower K_m for the phenylalanine- than the methionine-containing substrate and had k_{cat}/K_m values for these substrates 16- and 8.5-fold higher, respectively, than those determined for clipsin.

Preferential Hydrolysis of Brain β-APP by Clipsin—We began looking for potential endogenous substrates of clipsin by incubating rat cortical membranes with Mg^{2+} extract and measuring loss of the major protein bands following separation of the incubated samples by polyacrylamide gel electrophoresis. One protein doublet (Fig. 5A, *arrow*) out of 20 bands measured showed a 66% decrease following incubation with the clipsin-containing extract (Fig. 5A, lane 3). This decrease could be blocked by inclusion of the clipsin inhibitor MeO-Suc-Ala-Ala-Pro-boro-Phe-OH (Fig. 5A, *lane 4*) but not the elastase inhibitor MeO-Suc-Ala-Ala-Pro-boro-Val-OH (Fig. 5A, lane 5). No other bands varied more than 25%, and no protease activity endogenous to cortical membranes could be detected when comparing 37 °C incubated membranes with parallel samples kept on ice (Fig. 5A, lanes 1 and 2). Because the generation of β-amyloid from brain β-APP suggests a cleavage event characteristic of a chymotrypsin-like enzyme (Kang *et al.*, 1987; Abraham *et al.*, 1988), we next immuno-

TABLE I

Kinetic constants for the hydrolysis of tetrapeptide p-nitroanilide substrates by rat brain clipsin, human leukocyte cathepsin G, and bovine chymotrypsin

The kinetic constants were determined in 50 mM Hepes (pH 7.5), 1 mM EDTA, 0.5 M NaCl, 0.1% Brij 35, and 10% dimethyl sulfoxide at 50 °C. K_m values were determined by the method of Lineweaver and Burk. k_{cat} values were calculated using α-1-AChy-titrated values for total enzyme concentration. Kinetic values are an average drawn from three Lineweaver-Burk plot determinations.

Protease	Substrate (R:Suc-Ala-Ala-Pro) (R':MeO-Suc-Ala-Ala-Pro)	Substrate concentration range	K_m	k_{cat}	k_{cat}/K_m
		mM	*mM*	s^{-1}	M^{-1} s^{-1}
Clipsin	R-Phe-*p*-nitroanilide	0.1–2.0	0.82	47	57,000
	R'-Met-*p*-nitroanilide	0.1–2.0	0.21	1.1	5,200
Cathepsin G	R-Phe-*p*-nitroanilide	0.1–2.0	3.3	16	4,800
	R'-Met-*p*-nitroanilide	0.1–1.0	0.54	2.3	4,300
Chymotrypsin	R-Phe-*p*-nitroanilide	0.02–2.0	0.13	120	910,000
	R'-Met-*p*-nitroanilide	0.2–2.0	1.8	80	44,000

FIG. 5. Preferential degradation of rat brain membrane β-APP by clipsin *in vitro*. Mg^{2+} extract at 60% final (~4.2 nM clipsin final) was preincubated with Mg^{2+} extract buffer or a specific peptide boronic acid inhibitor in Mg^{2+} extract buffer for 10 min at 37 °C before combining with a crude adult rat brain membrane preparation (2.8 mg/ml final) for 1 h. The reaction was terminated with sodium dodecyl sulfate-containing sample buffer. *A*, proteins separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels and stained with Coomassie Blue. *Arrow* denotes protein doublet that was decreased 66% in the presence of Mg^{2+} extract, the only bands to vary more than 20% among the conditions. *B*, Western blot of a gel identical to A immunostained with an antiserum to the carboxyl-terminal domain of β-APP. The immunoreactive polypeptide triplet at 110–130 kDa represents specific recognition of β-APP (Siman *et al.*, 1989a). Both Coomassie Blue-stained bands and immunostained bands were quantified by peak area analysis using an LKB scanning laser densitometer. *Lane 1*, membrane preparation + Mg^{2+} extract buffer (no enzyme) at 4 °C; *lane 2*, membrane preparation + Mg^{2+} extract buffer at 37 °C; *lane 3*, membrane preparation + 60% Mg^{2+} extract at 37 °C; *lane 4*, membrane preparation + 60% Mg^{2+} extract + MeO-Suc-Ala-Ala-Pro-boro-Phe-OH (10 μM final concentration) at 37 °C; *lane 5*, membrane preparation + 60% Mg^{2+} extract + MeO-Suc-Ala-Ala-Pro-boro-Val-OH (10 μM final concentration) at 37 °C.

stained Western blots of these same test samples for β-APP using a polyclonal antiserum raised against a synthetic peptide corresponding to residues 676–695 of the β-APP carboxyl terminus (Fig. 5*B*). The clipsin-containing extract caused a 90% loss of β-APP immunoreactivity (Fig. 5*B*, *lane 3*), and this loss was specifically blocked by the clipsin inhibitor MeO-Suc-Ala-Ala-Pro-boro-Phe-OH (Fig. 5*B*, *lane 4*).

Ontogeny and Tissue Distribution of Clipsin—To determine the developmental period during which clipsin activity was highest in brain, we prepared in parallel Mg^{2+} extracts from forebrain of rats at different postnatal ages (Fig. 6). We found the peak of clipsin activity to occur at around postnatal day 5. No activity was found in alternate fractions generated during the preparation of Mg^{2+} extract, arguing against an altered compartmentalization of clipsin during development (data not shown). To quantify these changes in activity, the Mg^{2+} extract fractions from different aged brains were also assayed for Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide hydrolysis *in vitro*. The percent increase in absorbance occurring over 1 h is listed at the *bottom* of the appropriate *lanes*. These *in vitro* values correspond well to the enzymographic detection of clipsin activity and indicate that clipsin activity is very low in newborn and adult rat brain but experiences a dramatic 12–14-fold increase during the first 2 weeks after birth.

We also examined the distribution of activities similar to clipsin among different tissues to compare with what has been reported for other chymotrypsin-like proteases. Because many chymotrypsin-like proteases have apparent migration rates centering around M_r 25,000 (Barrett and McDonald, 1980;
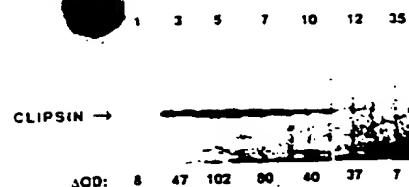
FIG. 6. Ontogeny of clipsin activity. Mg^{2+} extract was prepared in parallel from forebrain of rats, and the fractions were analyzed either by enzymography as in Fig. 2 (preincubation of gel strips in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA) or *in vitro* using 20% Mg^{2+} extract against 2 mM Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate for 1 h at 50 °C as in Fig. 4. An enzymograph of Mg^{2+} extracts prepared from forebrain of rats at various ages is shown focusing on the first 2 weeks postnatally. *Lanes* are identified at the *top* by postnatal age in days. To measure clipsin activity *in vitro*, absorbance was measured for each sample at 5 min and again at 65 min and normalized against a blank containing no enzyme. The value from the latter time point was divided by the value from the former time point and expressed as a percentage. The percent increase in absorbance (ΔOD) during the 1-h reaction is the percentage generated for each sample minus 100%. These values are listed at the *bottom* of the corresponding *panel* from the enzymographic analysis. The enzymographic analysis is representative of three independent experiments. The *in vitro* values are averages of three separate determinations.
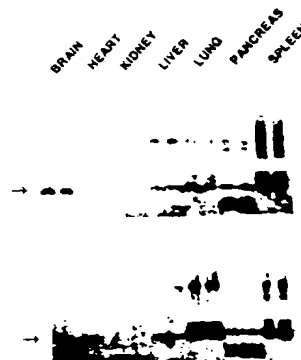
FIG. 7. Tissue distribution of activities similar to clipsin in physical properties. Mg^{2+} extracts were prepared from various tissues of 5-day-old and adult rat perfused with phosphate-buffered saline (see "Experimental Procedures"). These fractions were then analyzed by enzymography as in Fig. 1. Gels were preequilibrated in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA. *Upper panel*, enzymograph of tissues from 5-day-old rat. *Lower panel*, enzymograph of tissues from adult rat. Results are representative of two experiments.

Lorand, 1981), we prepared Mg^{2+} extracts from each tissue tested to select for proteases similar to clipsin with regard to solubilization properties (Fig. 7, *upper* and *lower panels*). Both 5-day-old (Fig. 7, *upper panel*) and adult (Fig. 7, *lower panel*) rat spleen were highest in an activity migrating close to clipsin's apparent molecular weight. Adult lung showed a dramatic developmental increase in a similar sized protease from 5-day-old to adult. Brain was distinguished as the only tissue having an M_r 25,000 activity that was more highly expressed at day 5 than in adult.

DISCUSSION

We have described a M_r 25,000 protease in rat brain which we have designated "clipsin" as an acronym for its identification as a chymotrypsin-like protease. Clipsin was inhibited by the general serine protease inhibitors phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate and not by inhibitors specific for the other three classes of protease. Within the serine class, clipsin was preferentially inhibited by MeO-Suc-Ala-Ala-Pro-boro-Phe, which is specific for chymotrypsin-like proteases (Kettner and Shenvi, 1984). Clipsin selec-

tively hydrolyzed Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide and MeO-Suc-Ala-Ala-Pro-Met-*p*-nitroanilide, two prototypical synthetic substrates of chymotrypsin-like proteases but could be distinguished from pancreatic and leukocyte elastase through its lack of inhibition by MeO-Suc-Ala-Ala-Pro-boro-Ala-OH and MeO-Suc-Ala-Ala-Pro-boro-Val-OH, respectively (Kettner and Shenvi, 1984) and its inability to hydrolyze the two *p*-nitroanilide counterparts of these tetrapeptides.

Both enzymography and an *in vitro* chromogenic substrate assay were used in parallel to characterize chymotrypsin-like activity in the clipsin-enriched Mg^{2+} extract. This approach allowed us to establish conditions under which clipsin activity as detected by enzymography was the only protease activity measured *in vitro*. Although it is possible that there is more than one chymotrypsin-like protease in the Mg^{2+} extract, this is argued against by: (*a*) the lack of any other protease activities on the enzymographs under the conditions used to assay clipsin; (*b*) the co-titration using α-1-AChy of chymotrypsin-like activity *in vitro* and clipsin activity on enzymographs; (*c*) the parallel ontogeny of brain chymotrypsin-like activity *in vitro* with the ontogeny of clipsin activity on enzymographs; and (*d*) the tight correspondence of *in vitro* and enzymographic inhibitor profiles. Clipsin also appears to be quite stable in Mg^{2+} extract as indicated by its linear substrate hydrolysis over 90 min or more *in vitro*.

Among chymotrypsin-like enzymes, clipsin could be kinetically distinguished from both chymotrypsin and cathepsin G. Clipsin resembled cathepsin G in terms of its greater relative affinity for the methionine- *versus* phenylalanine-containing *p*-nitroanilide substrate; however, clipsin had an 11-fold greater catalytic efficiency against Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide than MeO-Suc-Ala-Ala-Pro-Met-*p*-nitroanilide, whereas cathepsin G was equally reactive against the two substrates. It is unlikely that the kinetic differences between cathepsin G and clipsin were related to species differences in the enzyme because human and rat leukocyte cathepsin G have been reported to exhibit similar kinetic profiles (Virca *et al.*, 1984). Chymotrypsin was quite distinct kinetically from clipsin, having k_{cat}/K_m values 8.5-fold and 16-fold greater than clipsin for MeO-Suc-Ala-Ala-Pro-Met-*p*-nitroanilide and Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide, respectively. Because all three proteases were stable at higher temperatures, kinetic comparisons were performed at 50 °C to increase reaction rates (Barrett, 1981). Accordingly, our k_{cat} values are on the average 4.5-fold higher than those reported using lower reaction temperatures (Nakajima *et al.*, 1979).

Another group of well studied chymotrypsin-like enzymes is the rat mast cell proteases (RMCPs), also termed chymases (Powers *et al.*, 1985; Trong *et al.*, 1987). Although mast cells have been found in the central nervous system and constitute a potential source of protease activity associated with brain (Orr and Pace, 1984), they are restricted to surface leptomeninges in the neonatal rat central nervous system (Ibrahim, 1985), and these structures were removed prior to tissue homogenization in the present study. The narrow developmental window of clipsin activity also argues against a mast cell origin of clipsin because mast cell presence in the central nervous system steadily increases with development (Ibrahim, 1985). Although RMCP I and RMCP II were not directly compared with clipsin in the present study, they would appear to be kinetically distinct from clipsin. RMCP I and RMCP II have 5- and 3-fold greater affinities, respectively, for a phenylalanine- *versus* a methionine-containing tripeptide *p*-nitroanilide (Suc-Val-Pro-*X*-*p*-nitroanilide; Powers *et al.*, 1985). This stands in contrast to the nearly 4-fold greater affinity of clipsin for the methionine- *versus* phenylalanine-containing

tetrapeptide *p*-nitroanilide substrate used in the present study.

Clipsin was shown further to be a chymotrypsin-like enzyme in its property of binding to α-1-AChy in a sodium dodecyl sulfate-resistant manner. Our determination of clipsin's binding affinity for α-1-AChy ($k_{assoc} = 2.9 \times 10^6$ M^{-1} s^{-1}) is close to 2 orders of magnitude higher than the k_{assoc} reported for α-1-AChy binding to bovine chymotrypsin (6.0×10^4 M^{-1} s^{-1}) but is about 1 order of magnitude lower than that reported for human cathepsin G (5.1×10^7 M^{-1} s^{-1}; Beatty *et al.*, 1980). The demonstration that α-1-AChy stably binds to clipsin, aside from allowing the titration of enzyme present in our clipsin-enriched extract, also was important in that α-1-AChy or an α-1-AChy-like protein has been found in the normal human central nervous system (Justice *et al.*, 1987; Abraham *et al.*, 1989).

Cathepsin G is considered on the basis of its high k_{assoc} for α-1-AChy to be a primary target of α-1-AChy. Although the k_{assoc} found for clipsin binding to α-1-AChy in the present study is respectably high, it may still underestimate the binding affinity of clipsin for an α-1-AChy-like protein *in vivo* for two reasons: (*a*) although the α-1-AChy and cathepsin G used in these studies were both derived from human sources, clipsin was derived from rat tissue, offering a potential species-based mismatch and consequent decrease in catalytic efficiency; and (*b*) it is not yet known whether serum α-1-AChy and the α-1-AChy activity identified in brain represent identical enzymes. A brain-specific form of α-1-AChy may have a higher affinity for chymotrypsin-like proteases endogenous to that tissue.

Determining the tissue distribution of clipsin was complicated by its similarity to other chymotrypsin-like proteases with regard to molecular weight, substrate specificity, and solubilization in high magnesium. Proteases similar to and potentially identical to clipsin were present in other tissues, most notably adult lung and adult spleen, whereas lesser amounts were also detectable in liver and pancreas. Spleen has been reported to be particularly rich in cathepsin G and elastase (Starkey and Barrett, 1976). Lung showed a sizable developmental increase in activity of a protease(s) with identical mobility to the spleen protease. The brain protease was distinguished from similar molecular weight activities in other tissues by its presence in 5-day-old but not adult rats. In investigating this ontogeny more closely, we found that clipsin activity in brain was present in appreciable amounts only during a 12-day period from postnatal day 3 to day 14. Whether the decrease in activity after day 5 represents a decreased presence of the protein is not known. Although the enzymographic assay normally separates proteases from endogenous inhibitors prior to measuring their activity, this procedure does not dissociate sodium dodecyl sulfate-resistant protease/protease inhibitor complexes, such as were demonstrated to occur in the present report between clipsin and α-1-AChy. Potentially, then, the decrease in clipsin activity in brain after day 5 might not represent a decrease in clipsin expression but rather an increase in α-1-AChy levels and/or an increased access of α-1-AChy to clipsin. Such questions may be addressed using antibodies and nucleotide probes raised against both clipsin and α-1-AChy.

Brain α-1-AChy has received increasing attention due to its identification as an integral component of neuritic plaques in Down's syndrome and Alzheimer's disease (Abraham *et al.*, 1988). Isolation of neuritic plaques includes sodium dodecyl sulfate treatment to remove loosely associated proteins; however, the ability of α-1-AChy to form sodium dodecyl sulfate-resistant bonds with its target proteases invites the question

as to whether a chymotrypsin-like protease with α-1-AChy bound to it might also be an integral plaque component. Although α-1-AChy may potentially form sodium dodecyl sulfate-resistant bonds by an unknown mechanism to β-amyloid or another as yet unidentified protein, the recognized ability of α-1-AChy to form such bonds with clipsin and potentially other brain chymotrypsin-like proteases provides the most direct hypothesis needing to be investigated concerning the nature of α-1-AChy association with neuritic plaques.

A second line of evidence suggesting involvement of chymotrypsin-like enzymes in neuritic plaque formation lies in the generation of β-amyloid—the first identified neuritic plaque component—from the normal brain protein β-APP (Allsop et al., 1983; Glenner and Wong, 1984; Kang et al., 1987; Card et al., 1988; Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). The amino terminus in β-amyloid results from hydrolysis at the carboxyl end of methionine residue 596 (Kang et al., 1987). Such a cleavage event has been demonstrated at physiologically relevant rates only for the chymotrypsin-like proteases (Barrett and McDonald, 1980; Lorand, 1981), although such studies consider only primary protein structure. As a first step in determining potential endogenous substrates for clipsin, we tested the ability of clipsin to degrade major Coomassie Blue-detectable proteins from rat cortical membranes and, in parallel, examined degradation of β-APP from this same source using a carboxyl-terminal-directed antibody. β-APP proved to be an excellent substrate for clipsin, although the site(s) of cleavage cannot be determined from this analysis. We have found recently that several proteases, including calpain I, trypsin, and papain, are able to cleave β-APP.[3] Clipsin ranks with calpain in being extraordinarily selective for degradation of β-APP. Future studies will need to determine specifically whether clipsin or other brain chymotrypsin-like proteases can generate the amino terminus of β-amyloid from β-APP by: (a) testing their ability to hydrolyze synthetic peptides incorporating the sequence adjacent to Met[596] in β-APP; and (b) ultimately testing whether such proteases can perform this cleavage in the context of the entire β-APP.

The unusual ontogeny of clipsin activity in brain suggests that clipsin has a very circumscribed role that is largely constrained to a brief developmental period. It is tempting to speculate that inappropriate expression of clipsin activity outside this developmental period might lead to inappropriate processing of clipsin substrates such as β-APP. What could trigger an increase in activity? Recently, we reported that immunoreactivity of β-APP increases dramatically in reactive astrocytes following chemical or physical lesions in the brain (Siman et al., 1989a). Astrocytes normally show negligible β-APP staining (Card et al., 1988; Siman et al., 1989a). Similarly, gray matter surrounding damaged tissue in Alzheimer's disease and Down's syndrome exhibits numerous astrocytes positive for α-1-AChy immunostaining (Abraham et al., 1989) even though constitutive levels of α-1-AChy in the central nervous system are reported to be rather low (Justice et al., 1987). These observations suggest that these two proteins are produced by astrocytes in response to brain injury. To date,

clipsin has been detected in cultured primary astrocytes. Because proteases and their inhibitors are often co-released from cells (Eaton and Baker, 1983; Herron et al., 1986) it will be of interest to ascertain whether expression of clipsin activity in adult brain might be elevated in response to brain injury.

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(54) Title: β-SECRETASE, ANTIBODIES TO β-SECRETASE, AND ASSAYS FOR DETECTING β-SECRETASE INHIBITION

(57) Abstract

Compositions comprising a novel protease capable of cleaving β-amyloid precursor protein (APP) on the amino-terminal side of the β-amyloid peptide therein are provided. The protease is designated β-secretase. Reaction systems comprising β-secretase may be used in screening assays to monitor β-secretase modulated cleavage of APP and to identify β-secretase inhibitors, wherein the β-secretase is in the presence of a suitable polypeptide substrate and cleavage of the substrate is determined in the presence and absence of the test substance. Antibodies are raised against peptides of β-secretase. Pharmaceutical compositions and methods comprise compounds identified by screening assays.

β-SECRETASE, ANTIBODIES TO β-SECRETASE, AND ASSAYS FOR DETECTING β-SECRETASE INHIBITION

BACKGROUND OF THE INVENTION

1.   Field of the Invention

The present invention relates generally to the cleavage of β-amyloid precursor protein to produce β-amyloid peptide. More particularly, the present invention relates to isolated and purified compositions containing an enzyme responsible for such cleavage (β-secretase) and assays for identifying inhibitors of β-secretase.

Alzheimer's disease is characterized by the presence of numerous amyloid plaques and neurofibrillary tangles (highly insoluble protein aggregates) present in the brains of Alzheimer's disease patients, particularly in those regions involved with memory and cognition. β-amyloid peptide is a major constituent of amyloid plaque which is produced by cleavage of β-amyloid precursor protein. It is presently believed that a normal (non-pathogenic) processing of the β-amyloid precursor protein occurs via cleavage by a putative "α-secretase" which cleaves between amino acids 16 and 17 of the β-amyloid peptide region within the protein. It is further believed that pathogenic processing occurs in part via a putative "β-secretase" which cleaves at the amino-terminus of the β-amyloid peptide region within the precursor protein. Heretofore, however, the existence of β-secretase has not been confirmed.

The identification, isolation, and characterization of novel biological molecules having unique activities is generally useful. For example, novel enzymes can be used to catalyze reactions of a type associated with their class. In particular, novel proteases can be used to cleave proteins for a variety of purposes, and the availability of new proteases provides unique capabilities. In addition to such uses associated with enzymes in general, the identification,

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isolation, and purification of the putative β-secretase enzyme
would permit chemical modeling of a critical event in the
pathology of Alzheimer's disease and would allow the screening
of compounds to determine their ability to inhibit β-secretase
5   activity.

                For these reasons, it would be desirable to isolate,
purify, and characterize the enzyme responsible for the
pathogenic cleavage of β-amyloid precursor protein at the
amino-terminus of the β-amyloid peptide region. In
10   particular, it would be desirable to utilize such an enzyme
(referred to hereinafter as β-secretase) in methods for
screening candidate drugs for the ability to inhibit the
activity of β-secretase in in vitro systems. It would be
particularly desirable if such screening assays could be
15   performed in a rapid format which would permit the screening
of large numbers of test drugs in automated fashion.

2.   Description of the Background Art
                β-amyloid precursor protein (APP) is expressed in
20   three differently-spliced forms of 695, 751, and 770 amino
acids, and "normal" processing involves proteolytic cleavage
at a site between residues Lys^{16} and Leu^{17} in the β-amyloid
peptide. Kang et al. (1987) Nature 325:773-776. Soluble
β-amyloid peptide which has been cleaved at the putative
25   β-secretase site has also been found in the culture medium of
non-diseased cells (Haass et al. (1992) Nature 359:322-325)
and in CSF from healthy humans and animals (Seubert et al.
(1992) Nature 359:325-327). The possible existence of the
putative β-secretase is discussed in, for example, Selkoe,
30   "Cell Biology of the Amyloid β-Protein and the Mechanism of
Alzheimer's Disease," in Annual Review of Cell Biology,
Spudich et al., eds., Annual Review, Inc., Palo Alto,
California, vol. 10, 1994. The Swedish mutation of APP is
also discussed in Selkoe, supra. See also, Esch et al. (1994)
35   Science 248:1122.

SUMMARY OF THE INVENTION

The present invention provides novel β-secretase
compositions comprising an isolated and purified enzyme which
cleaves β-amyloid precursor protein (APP) at the amino-
terminus of β-amyloid peptide (βAP) within APP, referred to
hereinafter as "β-secretase activity." The compositions of
the present invention will generally have a β-secretase
activity which is at least five-fold greater than that of a
solubilized but unenriched membrane fraction from human 293
cells, preferably being at least ten-fold greater than that of
the membrane fraction, and more preferably being at least 100-
fold greater than that of the membrane fraction. The
β-secretase enzyme is characterized by (1) an apparent
molecular weight in the range from 260 kD to 300 kD as
determined by gel exclusion chromatography, (2) a more
accurate apparent molecular weight in the range from 60 kD to
148 kD determined by electrophoresis, (3) a net negative
charge at pH 5 and a net negative charge at pH 7.5, and (4)
binding to wheat germ agglutinin.

The compositions of the present invention are
generally useful as proteolytic chemicals and specifically
useful in assays for detecting proteolytic cleavage of APP
resulting from the novel β-secretase and determining whether a
test substance will inhibit such cleavage. The method
comprises exposing a polypeptide comprising the β-secretase
site of APP (located at the amino-terminus of the βAP region
within APP) to an at least partially purified β-secretase in
the presence of the test substance under conditions such that
the β-secretase would be expected to cleave the polypeptide
into an amino-terminal fragment and a carboxy-terminal
fragment in the absence of test substance which inhibits such
cleavage. Test substances which inhibit such cleavage may
then be introduced or exposed to the assay system to identify
which test substances have β-secretase inhibition activity.
Such test methods preferably employ the β-secretase
compositions described above. Generation of fragments of APP-
derived polypepetides is detected, e.g. by an antibody
specific for the carboxy end of the amino terminal fragment or

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the amino end of the carboxy-terminal fragment. The
polypeptide substrate for the β-secretase may comprise a
fusion polypeptide including an amino-terminal portion having
a binding epitope. Use of such a fusion polypeptide as the β-
5   secretase substrate facilitates detection of cleavage by
capture of the amino-terminal portion and labelling of the
amino-terminal portion.

The compositions will further comprise threshold
levels, typically at least 10% by weight, of enzymes which
10 cleave APP at the βAP cleavage site and which are reactive
with antibodies raised against immunogenic peptides of β-
secretase, such as any one or a combination of [SEQ ID No.:5],
[SEQ ID No.:6], and [SEQ ID No.:7].

The present invention still further provides
15 antibodies and antibody compositions that specifically bind to
β-secretase protein. The antibodies may be polycolonal or
monocolonal, and may be prepared by immunization of a suitable
host with any of the immunogenic β-secretase compositions
described above. The antibodies may further be prepared
20 recombinantly, may be humanized, or otherwise modified or
produced in accordance with conventional methods for antibody
production.

The present invention further provides methods and
assays for detecting β-secretase cleavage of a polypeptide
25 substrate, such as β-amyloid precursor protein (APP) or
synthetic or recombinant analogues thereof. The method
utilizes a reaction system including β-secretase and the
polypeptide substrate present in initial amounts. The
reaction system is maintained under conditions which permit
30 the β-secretase to cleave the polypeptide substrate into
cleavage products. The β-secretase cleavage reaction is
monitored by detecting the amount of at least one of the β-
secretase cleavage products, where the amount of cleavage
product(s) will increase over time as the reaction progresses.
35 Such methods are particularly useful for screening test
compounds for the ability to inhibit β-secretase activity.
Test compounds are introduced to the reaction system, and the
ability of the test compound to inhibit the β-secretase

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activity is determined based on the ability to decrease the
amount of cleavage product produced, usually in comparison to
a control where β-secretase mediated cleavage in the reaction
system is observed and measured in the absence of test
5   compound(s).

The reaction system may comprise β-secretase and
polypeptide substrate obtained from separate sources. For
example, β-secretase may be purified from a natural source or
be synthetically or recombinantly produced, as discussed in
10   detail hereinbelow. In such cases, the polypeptide substrate
may be full length APP, but will more usually be a shorter
polypeptide comprising the β-secretase cleavage site within
APP. The shorter polypeptide can be produced with label,
binding moiety, or other components which facilitate detection
15   in various assay protocols.

In an alternative assay format, both the β-secretase
and the polypeptide substrate will be obtained from a single
cellular source, e.g. cell membranes from brain cells or other
suitable sources. The cellular source will be treated to
20   release both the β-secretase and the polypeptide substrate
(which will be full length APP) into a suitable reaction
medium, where the conversion of APP into cleavage products may
be observed over time. Test compounds may be introduced to
the reaction system, and the ability of particular test
25   compounds to inhibit β-secretase activity determined generally
as described elsewhere herein.

The present invention further comprises methods for
inhibiting the cleavage of β-amyloid precursor protein (APP)
in cells. Such methods comprise administering to the cells an
30   amount of a compound effective to at least partially inhibit
β-secretase activity. Usually, such compounds will be
selected by the screening methods described above.

The present invention still further provides methods
for inhibiting the cleavage of β-amyloid precursor protein in
35   mammalian hosts. Such methods comprise administering to the
host an amount of a compound effective to inhibit β-secretase
activity in cells of the host, usually in brain cells of the
host. Such compounds will usually be selected by the

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screening assays described above.   Such methods will be useful
for treating conditions related to β-amyloid peptide
deposition such as Alzheimer's disease, Down's syndrome, and
the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a Western blot showing the reactivity of
antibodies raised against peptides Seek-1 [SEQ ID No.:5],
Seek-2 [SEQ ID No.:6], and Seek-3 [SEQ ID No.:7], under non-
reducing conditions, as described in the Experimental section.

Fig. 2 is a similar Western blot to Fig. 8, except
that the protein samples were reduced prior to
electrophoresis.

Fig. 3 is a chart comparing the immunoprecipitation
of β-secretase using the antibodies of Figs. 8 and 9 under
reducing and non-reducing conditions.

Fig. 4 is a schematic illustration of an APP-
containing fusion peptide useful as substrates in performing
the screening assays of the present invention, having a
binding epitope derived from maltose-binding protein (MBP).
An assay was run by exposing the fusion polypeptide to
β-secretase which cleaves the 125 amino acid portion of APP
(APP C-125) at the amino-terminus of the βAP. The MBP portion
may then be captured, and the carboxy-terminus of the APP
fragment which is exposed by cleavage with β-secretase may be
identified with 192 antibody specific for said terminus. SW-
192 antibody bound to a reporter is utilized, which antibody
recognizes the carboxy-terminus of the Swedish mutation of
APP.

Fig. 5 illustrates APP 638 which is a recombinantly
expressed form of APP truncated after βAP (Aβ). APP 638 may
be used in a β-secretase assay where the βAP peptide is
cleaved and the carboxy-terminus of the amino-terminal
fragment of APP 638 recognized by 192 antibody in either a
Western blot or ELISA assay. The carboxy terminal βAP
fragment can also be measured using a 3D6/266 assay.

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Fig. 6 is the complete nucleotide and amino acid sequence of the Swedish mutation of the fusion polypeptide of maltose-binding protein and APP fragment utilized in the Experimental section hereinafter [SEQ ID No.:1 and SEQ ID No.:17].

Fig. 7 is a standard curve generated for the β-secretase ELISA described in detail in the Experimental section below.

Fig. 8 is a standard curve for a β-secretase assay.

Fig. 9 show the results of a β-secretase assay using the curve of Fig. 8.

Fig. 10A, 10B, 10C, 10D and 10E are HPLC analyses of five β-secretase substrate peptides. Labels describe the indicated peptide, e.g. "Leu/Asp C-Term" denotes the C-terminal fragment of cleavage between Leu and Asp. In Figs. 10C-E, upper trace is the same as the lower trace, but magnified.

Figs. 11A, 11B, and 11C are summaries of β-secretase activity of GEC fractions, as measured by both peptide cleavage and ELISA assays.

Fig. 12 illustrates the results of a β-secretase inhibition assay using two test compounds (Congo Red and an inactive compound).

Fig. 13 is an autoradiogram showing the detection of β-secretase cleavage products in a cell membrane assay format.

Fig. 14 is a graph illustrating the extent of β-secretase cleavage of mature and immature APP over time.

Fig. 15 is a chart illustrating the extent of β-secretase cleavage of mature and immature APP over more extended time periods.

Fig. 16 illustrates the effect of putative β-secretase inhibitors of β-secretase cleavage of APP in the cell membrane assay.

Fig. 17 is a structural formula of a compound tested by the inhibition assay of the present invention, as described in the Experimental section below.

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DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a novel protease
which specifically cleaves the β-amyloid precursor protein
(APP) at the amino-terminus of the β-amyloid peptide (βAP)
5   therein. It is believed that this protease is the putative
β-secretase responsible for the pathogenic processing of APP
to produce βAP in βAP-related conditions, such as Alzheimer's
disease, Down's syndrome, HCHWA-D, and the like. Thus, the
novel protease of the present invention will be referred to
10   hereinafter as "β-secretase." The β-secretase of the present
invention will be useful as a protease in *in vitro* and *in vivo*
systems where proteases may generally find use. For example,
β-secretase may be used to cleave or attempt to cleave
proteins in order to characterize, process, modify, or
15   otherwise react with the protein as a substrate. Thus,
β-secretase will have general utility as a proteolytic
chemical reagent in a wide variety of chemical reactions and
systems. In addition, the β-secretase of the present
invention will have a specific utility in the performance of
20   screening assays to identify β-secretase inhibitors, *i.e.*,
test compounds which are able to inhibit the proteolytic
cleavage of APP in the presence of β-secretase. Such assays
will be described in detail below. In addition to the β-
secretase compositions and screening assay methods, the
25   present invention will further provide recombinant nucleic
acid molecules which encode at least a portion of β-secretase
and which are useful for a variety of purposes, including
expression of β-secretase, detection of β-secretase genes, and
the like. The present invention will still further provide
30   recombinantly produced β-secretase molecules and compositions,
usually by the expression of all or a portion of the β-
secretase gene. The present invention will still further
provide antibodies to epitopes on the native β-secretase
protein which are useful for screening and other assays.
35

1.   DEFINITIONS

Unless defined otherwise, all technical and
scientific terms used herein have the same meaning as commonly

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understood by those of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred

5   methods and materials are described. For purposes of the present invention, the following terms are defined below.

As used herein, "β-amyloid precursor protein" (APP) refers to a polypeptide that is encoded by a gene of the same name localized in humans on the long arm of chromosome 21 and

10   that includes a βAP region (defined below) within its carboxyl third. APP is a glycosylated, single-membrane-spanning protein expressed in a wide variety of cells in many mammalian tissues. Examples of specific isotypes of APP which are currently known to exist in humans are the 695-amino acid

15   polypeptide described by Kang et al. (1987) *Nature* 325:733-736. A 751-amino acid polypeptide has been described by Ponte et al. (1988) *Nature* 331:525-527 and Tanzi et al. (1988) *Nature* 331:528-530. A 770-amino acid isotype of APP is described in Kitaguchi et al. (1988) *Nature* 331:530-532. A

20   number of specific variants of APP have also been described having point mutations which can differ in both position and phenotype. A general review of such mutations is provided in Hardy (1992) *Nature Genet.* 1:233-234. A mutation of particular interest is designated the "Swedish" mutation where

25   the normal Lys-Met residues at positions 595 and 596 of the 695 form are replaced by Asn-Leu. This mutation is located directly upstream of the normal β-secretase cleavage site of APP, which occurs between residues 596 and 597 of the 695 form.

30   As used herein, "β-amyloid peptide" (βAP) refers to a family of peptides having lengths from 28 to 43 amino acids, with a common 43 amino acid form comprising residues 597-640 of the 695 amino acid isotype of APP. BAP is produced by processing of the APP including cleavage at both the amino-

35   terminus and carboxy-terminus of the region. It is believed that the β-secretase of the present invention is responsible for cleavage of APP at the amino-terminus of βAP in normal and pathogenic processing of APP in human cells.

10

As used herein, "specifically cleaves β-amyloid precursor protein (APP) at the β-amyloid peptide cleavage location" means that the β-secretase cleaves APP at only a single location and only at the site between amino acids 596

5   and 597 of the 695 isotype. Test for determining whether an enzyme possesses such specificity are described in the Experimental section hereinafter under the headings β-secretase Inhibitor Assays, Assays utilizing purified β-secretase and recombinant fusion peptide substrates. β-

10   secretase will cleave the MBP-C125 SW substrate at only the β-cleavage site and no other locations.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in

15   which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms "recombinant protein" and "recombinant β-secretase" refer to a protein that is produced by expression of a nucleotide

20   sequence encoding the amino acid sequence of the protein from a recombinant DNA molecule.

The terms "isolated" "purified" or "biologically pure" refer to material which is at least partially separated from and which is often substantially or essentially free from

25   components which normally accompany it as found in its native state. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein or nucleic acid molecule which is the predominant

30   protein or nucleic acid species present in a preparation is substantially purified. Generally, an isolated protein or nucleic acid molecule will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to represent greater than

35   90% of all macromolecular species present. More preferably the protein is purified to greater than 95%, and most preferably the protein is purified to essential homogeneity,

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wherein other macromolecular species are not detected by conventional techniques.

The term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically bind and recognize an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments products by digestion with various peptidases. This includes, e.g., Fab' and F(ab)'$_2$ fragments. The term "antibody, " as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies, and further includes "humanized" antibodies made by now conventional techniques.

The term "immunoassay" is an assay that utilizes an antibody to specifically bind an analyte. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the analyte.

A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available can be made detectible, e.g., by incorporating a radio-label into the peptide, and used to detect antibodies specifically reactive with the peptide). A label often generates a measurable signal, such as radioactivity, fluorescent light or enzyme activity, which can be used to quantitate the amount of bound label.

An amino acid sequence or a nucleotide sequence is "identical" to a reference sequence if the two sequences are the same when aligned for maximum correspondence over a comparison window. Optimal alignment of nucleotide and amino

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acid sequences for aligning comparison window may be
conducted by the local homology algorithm of Smith and
Waterman (1981) *Adv. Appl. Math.*, 2:482, by the homology
alignment algorithm of Needleman and Wunsch (1970) *J. Mol.
Biol.*, 48:443, by the search for similarity method of Pearson
and Lipman (988) *Proc. Natl. Acad. Sci.*, *U.S.A.* 85:2444, by
computerized implementations of these algorithms (GAP, BESFIT,
FASTA, and TFASTA in the Wisconsin Genetics Software Package
Release 7.0, Genetics Computer Group, 575 Science Dr.,
Madison, WI), or by inspection. The best alignment (i.e.,
resulting in the highest percentage of homology over the
comparison window, i.e., 150 or 200 amino acids) generated by
the various methods selected. The percentage of sequence
identity is calculated by comparing two optimally aligned
sequences over the window of comparison, determining the
number of positions at which the identical amino acid occurs
in both sequences to yield the number of matched positions,
dividing the number of matched positions by the total number
of positions in the window of comparison (i.e., the window
size), and multiplying the result by 100 to yield the
percentage of sequence identity.

An amino acid sequence or a nucleotide sequence is
"substantially identical" or "substantially similar" to a
reference sequence if the amino acid sequence or nucleotide
sequence has at least 80% sequence identity, at least 85%
sequence identity, at least 90% sequence identity, at least
95% sequence identity or at least 99% sequence identity with
the reference sequence over a comparison window. Two
sequences that are identical to each other are, of course,
also substantially identical. An indication that two peptides
have amino acid sequences that are substantially identical is
that one peptide is immunologically reactive with antibodies
raised against the second peptide. Thus, a polypeptide is
substantially identical to a second polypeptide, for example,
where the two peptides differ only by a conservative
substitution. An indication that two nucleotide sequences are
substantially identical is that the polypeptide which the
first nucleotide sequence encodes is immunologically cross-

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reactive with the polypeptide encoded by the second nucleotide
sequence. Another indication that two nucleotide sequences
are substantially identical is that the two nucleic acid
molecules hybridize to each other under stringent conditions.

5   Stringent conditions are sequence dependent and are different
under different environmental parameters. Generally,
stringent conditions are selected to be about 5° C to 20° C
lower than the thermal melting point (T_m) for the specific
sequence at a defined ionic strength and pH. The T_m is the

10   temperature (under defined ionic strength and pH) at which 50%.
of the target sequence hybridizes to a perfectly matched
probe.

        An antibody "specifically binds to" or "is
specifically immunoreactive with" a protein when the antibody

15   functions in a binding reaction which is determinative of the
presence of the protein in the presence of a heterogeneous
population of proteins and other biologics. Thus, under
designated immunoassay conditions, the specified antibodies
bind preferentially to a particular protein and do not bind in

20   a significant amount to other proteins present in the sample.
Specific binding to a protein under such conditions requires
an antibody that is selected for specificity for a particular
protein. A variety of immunoassay formats may be used to
select antibodies specifically immunoreactive with a

25   particular protein. For example, solid-phase ELISA
immunoassays are routinely used to select monoclonal
antibodies specifically immunoreactive with protein. See
Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold
Spring Harbor Publications, New York, for a description of

30   immunoassay formats and conditions that can be used to
determine specific immunoreactivity.

        As used herein, "test compounds" may be any
substance, molecule, compound, mixture of molecules or
compounds, or any other composition which is suspected of

35   being capable of inhibiting β-secretase activity in vivo or in
vitro. The test compounds may be macromolecules, such as
biological polymers, including proteins, polysacchrides,
nucleic acids, or the like. More usually, the test compounds

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will be small molecules having a molecular weight below about
2 kD, more usually below 1.5 kD, frequently below 1 kD, and
usually in the range from 100 to 1,000 D, and even more
usually in the range from 200 D to 750 D. Such test compounds
5   may be preselected based on a variety of criteria. For
example, suitable test compounds may be selected as having
known proteolytic inhibition activity. Alternatively, the
test compounds may be selected randomly and tested by the
screening methods of the present invention. Such test
10   compounds will typically be administered to reaction system
(as discussed hereinbelow) at a concentration in the range
from about 1 nM to 1 mM, usually from about 1 μM to 1 mM.
Test compounds which are able to inhibit β-secretase cleavage
of APP are considered as candidates for further screening of
15   their ability to decrease βAP production in cells and/or
animals.

II. $\underline{\beta\text{-Secretase}}$

        β-secretase has been characterized in a number of
20   respects, as described in detail in the Experimental section
below. β-secretase has an apparent molecular weight in the
range from 260 kD to 300 kD determined by gel exclusion
chromatography in 0.2% hydrogenated Triton X-100. A more
accurate molecular weight in the range from 60 kD to 148 kD
25   has been determined by electrophoresis. β-secretase will bind
to wheat germ agglutinin but not to concanavalin A. It has
been found to have a net negative charge at pH 5 (where it
does not bind to a cationic exchange material) and a net
negative charge at pH 7.5 (where it binds to an anion exchange
30   material). The β-secretase of the present invention will
cleave both wild-type (normal) and the Swedish mutation of APP
at the putative β-secretase cleavage site on the immediate
amino-terminal side of the βAP fragment, and has been found to
have a higher proteolytic activity with respect to the Swedish
35   form of APP. Proteolytic activity appears to be at its peak
at a pH from 5 to 5.5, with very low activity at pH 7.5 and
above. β-secretase is resistant to many known protease
inhibitors (see Table 3 in the Experimental section below).

15

β-secretase appears to preferably recognize only those
polypeptide substrates which have retained a substantial
number of residues upstream and downstream from the cleavage
site (from either the wild-type, Swedish, or other mutated
5   form) of APP. As demonstrated in the Experimental section
hereinafter, peptides containing as few as five residues
upstream and five residues downstream from the β-secretase
cleavage site will be cleaved, but require longer incubation
periods and higher enzyme levels than does cleavage of
10   peptides containing longer regions on either side of the
cleavage site.

The β-secretase of the present invention will be
provided in an isolated and purified form. By "isolated and
purified," it is meant that the β-secretase has been either
15   (1) isolated and at least partially purified from a natural
source, such as human brain tissue or human 293 cells (as
described in detail in the Experimental section below).
β-secretase can be obtained from cellular sources using known
protein purification techniques. Contaminating proteins may
20   be removed from the β-secretase compositions by specific
techniques, including serial lectin chromatography on agarose-
bound succinylated-wheat germ agglutinin (SWGA) and agarose-
bound lentil lectin (LCA). These lectins, although partly
binding β-secretase activity, preferentially bind other
25   contaminating proteins in the purified fractions, and thus
allow increased enrichment of the β-secretase activity. .
The β-secretase will be isolated and purified to an extent
sufficient to increase the β-secretase activity in the
resulting composition to a useful level. In particular, the
30   β-secretase preparations of the present invention will have
sufficient activity to cleave APP and APP-containing
polypeptides as described in the Experimental section below.
Preferably, the β-secretase compositions of the present
invention will have an activity which is at least 10-fold
35   greater than that of a solubilized but unenriched membrane
fraction from human 293 cells. More preferably, the
compositions will have a β-secretase activity which is at
least about 100-fold greater than that of the solubilized

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membrane fraction from human 293 cells. A specific method for determining β-secretase activity in units of "ng m^{-1} h^{-1}" is described in detail in the Experimental section below (see footnote 1 to Table 1).

5   This invention also provides β-secretase protein analogs. As used herein, the term "β-secretase protein analog" refers to a non-naturally occurring polypeptide comprising a contiguous sequence fragment of at least 10 amino acids, at least 15 amino acids, at least 20 amino acids or at

10   least 25 amino acids from the sequence of native β-secretase. In one embodiment, β-secretase protein analogs, when presented as an immunogen, elicit the production of an antibody which specifically binds to native β-secretase protein. β-secretase protein analogs optionally are in isolated form.

15   This invention also provides active β-secretase protein analogs that cleave β-amyloid precursor protein at the β-amyloid peptide cleavage location, i.e. at a location immediately amino-terminal to the β-amyloid peptide. These analogues will preferably have the minimum activities

20   described above.

          Active β-secretase protein analogs include β-secretase protein analogs whose amino acid sequence differs from that of native β-secretase by the inclusion of amino acid substitutions, additions or deletions (e.g., active

25   fragments). Active fragments can be identified empirically by proteolytically cutting back the protein from either the amino-terminus or the carboxy-terminus or by deleting internal sequences to generate fragments, and testing the resulting fragments for activity.

30   Active β-secretase protein analogs having additions include those having amino acid extensions to the amino- or carboxy-terminal end of other active fragments, as well as additions made internally to the protein.

          Protein analogs that are oligopeptides can be

35   prepared by chemical synthesis using well known methods. However, both oligopeptides and larger β-secretase proteins and protein analogs preferably are prepared recombinantly.

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The β-secretase polypeptides of the present invention may also have amino acid residues which have been chemically modified by known techniques, such as phosphorylation, sulfonation, biotinylation or the addition of

5   other moieties. In some embodiments, the modifications will be useful for labelling reagents, purification targets, affinity ligands targeting, or the like.

III. ANTIBODIES AND HYBRIDOMAS

10   The β-secretase polypeptides of the present invention may be used to prepare polyclonal and/or monoclonal antibodies using conventional techniques with the β-secretase polypeptides as an immunogen. The intact β-secretase molecule, or fragments thereof, optionally coupled to a

15   carrier molecule, may be injected into small vertebrates, with monoclonal antibodies being produced by well-known methods, as described in detail below. Antibodies produced from β-secretase will be useful for performing conventional immunoassays to detect β-secretase in biological and other

20   specimens. Antibodies according to the present invention will bind to β-secretase with an affinity of at least 10^6 M^{-1}, 10^7 M^{-1}, 10^8 M^{-1}, or 10^9 M^{-1}.

A number of immunogens can be used to produce antibodies that specifically bind β-secretase polypeptides.

25   Recombinant or synthetic polypeptides of 10 amino acids in length, or greater, are the preferred polypeptide immunogen for the production of monoclonal or polyclonal antibodies. Exemplary peptides include [SEQ ID Nos.:5, 6, and 7]. In one class of preferred embodiments, an immunogenic peptide

30   conjugate is also included as an immunogen. Naturally occurring polypeptides can also be used either in pure or impure form.

Recombinant polypeptides are expressed in eurkaryotic or prokaryotic cells and purified using standard

35   techniques. The polypeptide, or synthetic version thereof, is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies can be generated

for subsequent use in immunoassays to measure the presence and quantity of the polypeptide.

Methods of producing polyclonal antibodies are known to those of skill in the art. In brief, an immunogen,
5   preferably a purified polypeptide, a polypeptide coupled to an appropriate carrier (e.g., GST, keyhole limpet hemanocyanin, etc.) or a polypeptide incorporated into an immunization vector such as a recombinant vaccinia virus (see, U.S. Patent No, 4, 722,848) is mixed with an adjuvant and animals are
10   immunized with the mixture. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When approximately high titers of antibody to the immunogen are obtained, blood is collected from the animal and
15   antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired. See e.g., Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor
20   Press, NY.

Antibodies, including binding fragments and single chain recombinant versions thereof, against predetermined fragments of β-secretase proteins are raised by immunizing animals, e.g., with conjugates of the fragments with carrier
25   proteins as described above. Typically, the immunogen of interest is a peptide of at least 3 amino acids, more typically the peptide is 5 amino acids in length, preferably, the fragment is 10 amino acids in length and more preferably the fragment is 15 amino acids in length or greater. The
30   peptides can be coupled to a carrier protein (e.g., as a fusion protein), or are recombinantly expressed in an immunization vector. Antigenic determinants on peptides to which antibodies bind are typically 3 to 10 amino acids in length.

35   Monoclonal antibodies may be prepared from cells secreting the desired antibody. In some instances, it is desirable to prepare monoclonal antibodies from particular mammalian hosts, such as mice, rodents, primates, humans, etc.

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Description of techniques for preparing such monoclonal
antibodies are found in, e.g., Stites et al. (eds.) *Basic and*
Clinical Immunology (4th ed.) Lange Medical Publications, Los
Altos, CA, and references cited therein; Harlow and Lane,
5   *Supra*; Goding (1986) *Monoclonal Antibodies: Principles and*
Practice (2d ed.) Academic Press, New York, NY; and Kohler and
Milstein (1975) *Nature* 256:495-497. Summarized briefly, this
method proceeds by injecting an animal with an immunogen. The
animal is then sacrificed and cells taken from its spleen,
10   which are fused with myeloma cells. The result is a hybrid
cell or "hybridoma" that is capable of reproducing *in vitro*.
The population of hybridomas is then screened to isolate
individual clones, each of which secrete a single antibody
species to the immunogen. In this manner, the individual
15   antibody species obtained are the products of immortalized and
cloned singe B cells from the immune animal generated in
response to a specific site recognized on the immunogenic
substance.
        Alternative methods of immortalization include
20   transformation with Epstein Barr Virus, oncogenes, or
retroviruses, or other methods known in the art. Colonies
arising from single immortalized cells are screened for
production of antibodies of the desired specificity and
affinity for the antigen, and yield the monoclonal antibodies
25   produced by such cells is enhanced by various techniques,
including injection into the peritoneal cavity of a vertebrate
(preferably mammalian) host. The polypeptides and antibodies
of the present invention are not with or without modification,
and include chimeric antibodies such as humanized murine
30   antibodies.
        Other suitable techniques involve selection of
libraries of recombinant antibodies in phage or similar
vectors. See, Huse et al. (1989) *Science* 246: 1275-1281; and
Ward, et al. (1989) *Nature* 341: 544-546.
35   Frequently, the polypeptides and antibodies will be
labeled by joining, either covalently or non covalently, a
substance which provides for a detectable signal. A wide
variety of labels and conjugation techniques are know and are

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reported extensively in both the scientific and patent
literature. Suitable labels include radionucleotides,
enzymes, substrates, cofactors, inhibitors, fluorescent
moieties, chemiluminescent moieties, magnetic particles, and

5   the like. Patents teaching the use of such labels include
U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345;
4,277,437; 4,272,149; and 4,366,241. Also, recombinant
immunoglobulins may be produced. See, Cabilly, U.S. Patent
No. 4,816,567; and Queen et al. (1989) *Proc. Natl'l Acad. Sci.*

10   *USA* 86: 10029-10033.

The antibodies of this invention are also used for
affinity chromatography in isolating β-secretase proteins.
Columns are prepared, e.g., with the antibodies linked to a
solid support, e.g., particles, such as agarose, Sephadex, or

15   the like, where a cell lysate is passed through the column,
washed and treated with increased concentrations of a mild
denaturant, whereby purified β-secretase polypeptides are
released.

The antibodies can be used to screen expression
20   libraries for particular expression products such as mammalian
β-secretase. usually the antibodies in such a procedure are
labeled with a moiety allowing easy detection of presence of
antigen by antibody binding.

Antibodies raised against β-secretase can also be
25   used to raise anti-idiotypic antibodies. These are useful for
detecting or diagnosing various pathological conditions
related to the presence of the respective antigens.

An alternative approach is the generation of
humanized immunoglobulins by linking the CDR regions of the
30   non-human antibodies to human constant regions by recombinant
DNA techniques. See Queen et al., *Proc. Natl. Acad. Sci. USA*
86:10029-10033 (1989) and WO 90/07861. The humanized
immunoglobulins have variable region framework residues
substantially from a human immunoglobulin (termed an acceptor
35   immunoglobulin) and complementarity determining regions
substantially from a mouse immunoglobulin, (referred to as the
donor immunoglobulin). The constant region(s), if present,
are also substantially from a human immunoglobulin. The human

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variable domains are usually chosen from human antibodies
whose framework sequences exhibit a high degree of sequence
identity with the murine variable region domains from which
the CDRs are derived. The heavy and light chain variable

5   region framework residues can be derived from the same or
different human antibody sequences. The human antibody
sequences can be the sequences of naturally occurring human
antibodies or can be consensus sequences of several human
antibodies. See Carter et al., WO 92/22653. Certain amino

10   acids from the human variable region framework residues are
selected for substitution based on their possible influence on
CDR conformation and/or binding to antigen. Investigation of
such possible influences is by modeling, examination of the
characteristics of the amino acids at particular locations, or

15   empirical observation of the effects of substitution or
mutagenesis of particular amino acids.

For example, when an amino acid differs between a
murine variable region framework residue and a selected human
variable region framework residue, the human framework amino

20   acid should usually be substituted by the equivalent framework
amino acid from the mouse antibody when it is reasonably
expected that the amino acid:

(1)   noncovalently binds antigen directly,

(2)   is adjacent to a CDR region,

25   (3)   otherwise interacts with a CDR region (e.g., is
within about 3 Å of a CDR region), or

(4)   participates in the V_L-V_H interface.

Other candidates for substitution are acceptor human
framework amino acids that are unusual for a human

30   immunoglobulin at that position. These amino acids can be
substituted with amino acids from the equivalent position of
the antibody or from the equivalent positions of more typical
human immunoglobulins.

A further approach for isolating DNA sequences which

35   encode a human monoclonal antibody or a binding fragment
thereof is by screening a DNA library from human B cells
according to the general protocol outlined by Huse et al.,
Science 246:1275-1281 (1989) and then cloning and amplifying

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the sequences which encode the antibody (or binding fragment) of the desired specificity. The protocol described by Huse is rendered more efficient in combination with phage display technology. See, e.g., Dower et al., WO 91/17271 and

5   McCafferty et al., WO 92/01047. Phasge display technology can also be used to mutagenize CDR regions of antibodies previously shown to have affinity for β-secretase protein receptors or their ligands. Antibodies having improved binding affinity are selected.

10   In another embodiment of the invention, fragments of antibodies against β-secretase protein or protein analogs are provided. Typically, these fragments exhibit specific binding to the β-secretase protein receptor similar to that of a complete immunoglobulin. Antibody fragments include separate

15   heavy chains, light chains Fab, Fab', F(ab')$_2$, Fabc, and Fv. Fragments are produced by recombinant DNA techniques, or by enzymic or chemical separation of intact immunoglobulins.

IV.   SCREENING ASSAYS

20   The present invention further provides assays for detecting β-secretase mediated cleavage of APP and other polypeptides substrates recognized by β-secretase. The methods utilize a reaction system which includes both a β-secretase component and a substrate component, where the β-

25   secretase cleaves the substrate over time to produce cleavage products. Thus, β-secretase activity can be observed and monitored over time as the amount of cleavage product(s) increases. The amount of cleavage product(s) in the reaction system can be measured in a variety of ways, including

30   immunologic, chromatographic, electrophoretic, and the like.
            Such β-secretase cleavage methods are particularly useful for screening test compounds to determine their ability to inhibit β-secretase mediated cleavage of APP. In such cases, a test compound is added to the reaction system and the

35   effect of the test compound on production of cleavage product is observed. Those compounds which inhibit the production of cleavage product(s) are considered as potential β-secretase

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inhibitors and further as potential therapeutic agents for treatment of conditions associated with βAP production.

The reaction system will usually comprise one of two types. First, the reaction system may comprise a β-secretase
5   and a polypeptide substrate which are obtained separately from different sources and thereafter admixed into the reaction mixture. Usually, the β-secretase will be either a purified or partially purified β-secretase obtained from a cellular source, as described above, or will be a recombinant β-
10   secretase, also as described above. The polypeptide substrate, in turn, will usually be either full length APP isolated from a natural source or produced recombinantly, a fragment of APP or other polypeptide which mimics a portion of APP and comprises the β-secretase cleavage site (as described
15   in more detail below), or a synthetic peptide comprising the β-secretase cleavage site. The β-secretase and polypeptide substrate can be used in a wide variety of solid phase detection systems which permit observance of the production of β-secretase cleavage products over time.
20   Alternatively, the reaction system may comprise native β-secretase and native APP obtained from a single, common cellular source, usually being simultaneously extracted from cell membranes. As described in more detail in the Experimental section hereinafter, human brain or other cells
25   may be obtained from culture, disrupted, and treated to obtain supernatants which comprise both β-secretase and native APP in amounts which permit subsequent conversion of the APP into cleavage products by the β-secretase. The cleavage products may be detected in the same way as described elsewhere in the
30   present application, and the methods will be particularly useful for determining the ability of test compounds to inhibit such β-secretase mediated cleavage.

The first β-secretase assay described above may be performed by combining an at least partially purified
35   β-secretase is combined with a polypeptide substrate comprising the β-secretase cleavage site of APP in the presence of the test substrate. Conditions are maintained such that the β-secretase would cleave the polypeptide

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substrate into an amino-terminal fragment and a carboxy-terminal fragment in the absence of a substance which inhibits such cleavage. Cleavage of the polypeptide substrate in the presence of the test compound is compared with that in the absence of the test compound, and those test substances which provide significant inhibition of the cleavage activity (usually at least about 25% inhibition, more usually at least about 50% inhibition, preferably at least about 75% inhibition, and often at least about 90% inhibition or higher) are considered to be β-secretase inhibitors. Such β-secretase inhibitors may then be subjected to further *in vitro* and/or *in vivo* testing to determine if they inhibit the production of βAP in cellular and animal models. Suitable *in vivo* and *in vitro* tests are described in copending application Serial Nos. 07/965,972 and 07/831,722, the full disclosures of which are incorporated herein by reference.

Suitable substrate polypeptides will include a region of the APP molecule which is recognized and cleaved by β-secretase. Usually, the substrate polypeptide will include at least about 5 amino acid residues, and preferably at the least about 17 amino acids, amino-terminal to the cleavage site (located between amino acids 596 and 597 in the 695-amino acid APP isomer) and at least about 5 amino acids, preferably at least about 16 amino acids, and most preferably at least 42 amino acids (i.e., the full βAP sequence), on the carboxy-terminal side of the cleavage site. The cleavage site will typically comprise the Met-Asp or the Leu-Asp cleavage site characteristic of the wild-type and Swedish forms of βAPP. An intact APP molecule will be suitable as the polypeptide including both wild-type and mutant forms of APP, particularly including the Swedish mutation of APP. Use of fusion substrate polypeptides is often preferred, where an affinity region can be fused to the β-secretase cleavage site of APP, producing a molecule whose cleavage can be conveniently monitored in solid phase test systems.

The screening assays of β-secretase and suitable substrate polypeptide are conveniently performed using "sandwich" assays where the amino-terminal or the carboxy-

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terminal fragment produced by cleavage is captured on a solid
phase. The captured fragment may then be detected using an
antibody specific for the end of the fragment exposed by
β-secretase cleavage. In an exemplary embodiment described in
5   detail in the Experimental section below, the polypeptide
substrate is a fusion polypeptide combining maltose-binding
protein and a 125-amino acid carboxy-terminal fragment of APP.
The assay uses anti-maltose-binding protein antibody to
capture the amino-terminal cleavage product, where the
10   carboxy-terminus of the cleavage product is detected by an
antibody specific thereto. An exemplary antibody is 192
antibody or SW-192 antibody, described in more detail in
copending application 08/143,697, filed on October 27, 1993,
the full disclosure of which is incorporated herein by
15   reference. The binding of the antibody to the cleaved fusion
polypeptide is detected using conventional labelling systems,
such as horseradish peroxidase or other detectable enzyme
labels, which are bound to the antibody directly (covalently),
or indirectly through intermediate linking substances, such as
20   biotin and avidin.

V.   Pharmaceutical Compositions and Therapeutic Methods
         The present invention further comprises methods for
inhibiting the β-secretase mediated cleavage of APP to APP
25   cleavage products in cells, where the method comprises
administering to the cells compounds selected by the method
described above. The compounds may be added to cell culture
in order to inhibit APP cleavage which results in βAP
production of other cultured cells. The compounds may also be
30   administered to a patient in order to inhibit β-secretase
mediated APP cleavage which results in pathogenic βAP
production and the deposition of amyloid β-plaque associated
with Alzheimer's Disease and other βAP-related conditions.
         The present invention further comprises
35   pharmaceutical compositions incorporating a compound selected
by the above-described method and including a pharmaceutically
acceptable carrier. Such pharmaceutical compositions should
contain a therapeutic or prophylactic amount of at least one

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compound identified by the method of the present invention.
The pharmaceutically acceptable carrier can be any compatible,
non-toxic substance suitable to deliver the compounds to an
intended host. Sterile water, alcohol, fats, waxes, and inert
5   solids may be used as the carrier. Pharmaceutically
acceptable adjuvants, buffering agents, dispersing agents, and
the like may also be incorporated into the pharmaceutical
compositions. Preparation of pharmaceutical conditions
incorporating active agents is well described in the medical
10   and scientific literature. See, for example, Remington's
Pharmaceutical Sciences, Mack Publishing Company, Easton,
Pennsylvania, 16th Ed., 1982, the disclosure of which is
incorporated herein by reference.

        The pharmaceutical compositions just described are
15   suitable for systemic administration to the host, including
both parenteral and oral administration. The pharmaceutical
compositions will usually be administered parenterally, i.e.
subcutaneously, intramuscularly, or intravenously. Thus, the
present invention provides compositions for administration to
20   a host, where the compositions comprise pharmaceutically
acceptable solution of the identified compound in an
acceptable carrier, as described above.

        Frequently, it will be desirable or necessary to
introduce the pharmaceutical compositions directly or
25   indirectly to the brain. Direct techniques usually involve
placement of a drug delivery catheter into the host's
ventricular system to bypass the blood-brain barrier.
Indirect techniques, which are generally preferred, involve
formulating the compositions to provide for drug latentiation
30   by the conversion of hydrophilic drugs into lipid-soluble
drugs. Latentiation is generally achieved through blocking of
the hydroxyl, carboxyl, and primary amine groups present on
the drug to render the drug more lipid-soluble and amenable to
transportion across the blood-brain barrier. Alternatively,
35   the delivery of hydrophilic drugs can be enhanced by intra-
arterial infusion of hypertonic solutions which can
transiently open the blood-brain barrier.

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The concentration of the compound in the pharmaceutical carrier may vary widely, i.e. from less than about 0.1% by weight of the pharmaceutical composition to about 20% by weight, or greater. Typical pharmaceutical

5   composition for intramuscular injection would be made up to contain, for example, one to four ml of sterile buffered water and one μg to one mg of the compound identified by the method of the present invention. The typical composition for intravenous infusion could be made up to contain 100 to 500 ml

10 of sterile Ringer's solution and about 1 to 100 mg of the compound.

The pharmaceutical compositions of the present invention can be administered for prophylactic and/or therapeutic treatment of diseases related to the deposition of

15 βAP, such as Alzheimer's disease, Down's syndrome, and advanced aging of the brain. In therapeutic applications, the pharmaceutical compositions are administered to a host already suffering from the disease. The pharmaceutical compositions will be administered in an amount sufficient to inhibit

20 further deposition of βAP plaque. An amount adequate to accomplish this defined as a "therapeutically effective dose." Such effective dose will depend on the extent of the disease, the size of the host, and the like, but will generally range from about μg to 10 mg of the compound per kilogram of body

25 weight of the host, with dosages of 0.1 μg to 1 mg/kg being more commonly employed.

For prophylactic applications, the pharmaceutical compositions of the present invention are administered to a host susceptible to the βAP-related disease, but not already

30 suffering from such disease. Such hosts may be identified by genetic screening and clinical analysis, as described in the medical literature (e.g. Goate (1991) Nature 349:704-706). The pharmaceutical compositions will be able to inhibit or prevent deposition of the βAP plaque at a symptomatically

35 early stage, preferably preventing even the initial stages of the β-amyloid disease. The amount of the compound required for such prophylactic treatment, referred to as a

prophylactically-effective dosage, is generally the same as
described above for therapeutic treatment.

    The following examples are offered by way of
illustration, not by way of limitation.

EXPERIMENTAL

Purification and Characterization of β-Secretase

    Frozen tissue (293 cell paste or human brain) was
cut into pieces and combined with five volumes of
homogenization buffer (20 mM Hepes, pH 7.5, 0.25 M sucrose,
2 mM EDTA). The suspension was homogenized using a blender
and centrifuged at 1000 x g (10 min, 4°C) to produce a post-
nuclear supernatant which was saved on ice. The pellets were
resuspended in fresh homogenizing buffer at the original
volume, and the centrifugation step was repeated. The second
supernatant was combined with the first one, and the
supernatant pool ("PNS") was centrifuged at 16,000 x g for
30 min at 4°C. The supernatants were discarded and the
pellets, labelled "P2," were either used immediately for
enzyme purification or frozen at -40°C for later use.

    The pellets were suspended in extraction buffer
(20 mM MES, pH 6.0, 0.5% Triton X-100, 150 mM NaCl, 2 mM EDTA,
5 μg/ml leupeptin, 5 μg/ml E64, 1 μg/ml pepstatin, 0.2 mM
PMSF) at the original volume. After vortex-mixing, the
extraction was completed by agitating the tubes at 4°C for a
period of one hour. The mixtures were centrifuged as above at
16,000 x g, and the supernatants were pooled. The pH of the
extract was adjusted to 7.5 by adding ~1% (v/v) of 1 M Tris
base (not neutralized).

    The neutralized extract was loaded onto a wheat germ
agglutinin-agarose (WGA-agarose) column pre-equilibrated with
10 column volumes of 20 mM Tris, pH 7.5, 0.5% Triton X-100,
150 mM NaCl, 2 mM EDTA, at 4°C. One milliliter of the agarose
resin was used for every 4 g of original tissue used. The
WGA-column was washed with 10 column volumes of the
 quilibration buffer, and then eluted as follows. Three-
quarter column volumes of 1 M N-acetylglucosamine in 20 mM
Tris, pH 7.5, 0.5% Triton X-100, 2 mM EDTA were passed through

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the column after which the flow was stopped for fifteen
minutes. An additional five column volumes of the 1 M
N-acetylglucosamine elution buffer were then used to elute the
column, followed by five column volumes of 10% chitin
5   hydrolysate in 20 mM Tris, pH 7.5, 0.5% Triton X-100, 2 mM
EDTA. All of the above eluates were combined (pooled WGA-
eluate).

The pooled WGA-eluate was diluted 1:4 with 20 mM
NaOAc, pH 5.0, 0.5% Triton X-100, 2 mM EDTA. The pH of the
10   diluted solution was adjusted to 5.0 by adding a few drops of
glacial acetic acid while monitoring the pH. This "SP load"
was passed through a 5-ml Pharmacia HiTrap SP-column
equilibrated with 20 mM NaOAc, pH 5.0, 0.5% Triton X-100, 2 mM
EDTA, at 4 ml/min at 4°C. β-Secretase activity was present in
15   the flow-through fraction, which was neutralized by adding
enough 1 M Tris (not neutralized) to bring the pH up to 7.5.
The enzyme solution was then loaded onto a 1-ml Pharmacia
HiTrap Q-column equilibrated with approximately 10 column
volumes of 20 mM Tris, pH 7.5, 0.2% hydrogenated Triton X-100,
20   2 mM EDTA, at 1.5 ml/min at 4°C. The column was washed with
10 column volumes of 20 mM Tris, pH 7.5, 0.2% hydrogenated
Triton X-100, 50 mM NaCl, 2 mM EDTA. Protein was eluted using
a linear gradient from 50 mM TO 350 Mm NaCl over 30 minutes at
a flow-rate of 1 ml/min at 4°C. The protein concentrations in
25   the HiQ fractions were measured using a BioRad colorimetric
protein assay, and the β-secretase activity was measured using
the MBP-C125 cleavage assay at pH 5.5. The fractions in the
ascending portion of the protein peak have the highest
specific activity and were pooled for further purification of
30   the enzyme.

The pooled fractions from the HiTrap Q were then
applied to a column of concanavalin A-agarose (10% v/v of
pool) equilibrated with 10 column volumes of 20 mM Tris,
pH 7.5, 0.2% hydrogenated Triton X-100, 150 mM NaCl, 2 mM
35   EDTA. The Con A flow-through was then loaded onto a
Superdex 200 (26/60) gel exclusion chromatography column,
which was eluted with Tris buffered saline, pH 7.4, 0.2%
hydrogenated Triton X-100, 2 mM EDTA, at 1 ml/min, collecting

3 min/fraction. Fractions containing β-secretase activity
were identified using the MBP-C125 cleavage assay. The
apparent molecular weight of the β-secretase activity eluting
from the Superdex column was estimated from the peak elution
volume (relative to that of standard proteins) to be
280,000 ± 9800 (average of two runs for 293 cells, and two
runs for human brain).

Results from a large-scale preparation of the enzyme
from human brain tissue is shown in Table 1 below.

Table 1

Step	Activity ng/ml/h	Protein µg/ml	Sp. Act.[1] ng/ml/h/µg protein	Fold Purfn.
Solubilized membrane extr.	2700	350	7.7	1
HiQ Elution pool	80000	210	380.9	49.5
Con A Flow-Thru	80000	100	800	103.8
Superdex peak fraction	57000	< 5	> 11400	> 1480.5

[1] Specific activity of the purified β-secretase was
measured as follows. MBP C125-SW (described below) was
combined at approximately 0.7 µg/ml in 100 mM sodium
acetate, pH 5.5, with 0.3% Triton X-100. The amount of
product generated was measured by the β-secretase assay,
also described below. Specific activity was then
calculated as:

$$Sp.\,Act. = \frac{(Product\ conc.\ ng/ml)(Dilution\ factor)(Incubation\ vol.\ \mu l)}{(Enzyme\ sol.\ vol.\ \mu l)(Incubation\ time\ h.)(Enzyme\ conc.\ \mu g/ml)}$$

The Sp. Act. is thus expressed as ng of protein produced
per µg of β-secretase per hour.

Glycosylation of β-secretase has been investigated
using various immobilized lectins, and ability of
substantially purified β-secretase activity to bind to them
was determined. Table 2 summarizes this data. A "-" sign

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signifies less than 20% binding, "+" between 24-40% binding, "++" between 50-75% binding, and "+++" > 75% binding.

<u>Table 2</u>

<u>Lectin</u>	<u>β-Secretase Binding</u>
jequirity bean (APA)	+
jack bean (con A)	+
scotch broom (CSA)	+
jimson weed (DSA)	+
coral tree (ECA)	-
grifornia simplicifolia I	-
grifornia simplicifolia II	-
Jacalin (AIA)	+
lentil (LCA)	+
horseshoe crab (LPA)	-
tomato (LEL)	+
maackia (MAA)	+
peanut (PNA)	+
pokeweed (POA)	-
castor bean (RCA1)	-
potato (STL)	-
wheat germ - succinylated (SWGA)	+
China gourd (TKA)	+
stinging nettle (UDA)	+
gorse (UEAI)	-
gorse (UEAII)	-
hairy vetch (VVL)	-
wheat germ (WGA)	+++

Only a single lectin (WGA) bound β-secretase activity quantitatively, out of the many tested. Partial binding of

the activity (25-40%) to a number of other lectins probably
indicates heterogeneous glycosylation.

β-secretase purified as described was assayed in the
presence of a number of common protease inhibitors as follows.
5   Enzyme solution was mixed with the inhibitor, as described
below in the β-Secretase Inhibitor Assay section, and assayed
for activity remaining as a percentage of a control solution
incubated under otherwise identical conditions. IC_{50} values,
if any, were determined as the concentration of inhibitor
10   which resulted in 50% inhibition of the control activity. The
results are set forth in Table 3.

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Table 3

Inhibitor	Max Conc	IC50
SERINE PROTEASES		
aminoethylbenzene-sulfonyl fluoride	0.8 mM	NI
chymostatin	0.2 mM	NI
3,4-dichloroisocoumarin	0.5 mM	< 25% inh.
diisopropylfluoro-phosphate	2 mM	NI
elastatinal	0.2 mM	NI
phenylmethylsulfonyl-fluoride	1.0 mM	NI
CYSTEINE PROTEASES		
E-64	0.14 mM	NI
N-ethylmaleimide	10 mM	NI
iodoacetamide	10 mM	NI
METALLOPROTEASES		
EDTA	2 Mm	NI
phosphoramidon	10 mM	NI
o-phenanthroline		7 mM
m-phenanthroline		7 mM
ASPARTYL PROTEASES		
pepstatin	25 µM	NI
diazoacetylnorleucyl-methyl ester		> 5 mM
DIVALENT METAL IONS		
Cu		2 mM
Zn		3 mM
Hg		< 10% inh
Ca		NI
Mg		NI

These results indicate that β-secretase activity is not inhibited by common inhibitors of serine, cysteine, aspartyl, and metalloproteases. Although o-phenanthroline inhibits poorly, m-phenanthroline, which is not a metal chelator, also does so, suggesting that this weak inhibition is unrelated to the metal-chelating properties of o-phenanthroline.

Partial Sequencing, Production of Synthetic Peptides, and Production of Antibodies

The Superdex elution fractions containing the peak of β-secretase activity described above in connection with Table 1 were pooled and passed through a 1-ml succinylated wheat germ agglutinin agarose (SWGA-agarose, Vector Labs) column. The SWGA column had been previously washed with 5 column volumes of Tris-buffered saline, pH 7.4, 0.2% hydrogenated Triton X-100, 1 mM CaCl2, 1 mM MgCl2, followed by 5 column volumes of the same buffer with 1 M NaCl, and finally 10 column volumes of the first, low [NaCl] buffer. After the enzyme sample had been passed through the SWGA column, the resin was washed with an additional one-half column volume of the equilibration buffer. The flow through from this was pooled with the sample flow-through containing the bulk of the β-secretase activity.

The SWGA-agarose flow-through was then passed through a 1 ml lentil lectin agarose column (LCA-agarose, lens culinaris agglutinin, Vector Labs) washed and equilibrated as described above for the SWGA resin. The majority of the β-secretase activity is again recovered in the LCA flow-through fraction.

The LCA flow-through was then diluted 1:4 with 20 mM Tris, pH 7.5, 2 mM EDTA, 0.2% hydrogenated Triton X-100, and allowed to bind to 40 μl of DEAE-Sepharose Fast Flow (Pharmacia), by mixing the enzyme solution with the anion-exchange resin at 4oC overnight, with gentle agitation. The DEAE-Sepharose resin is then recovered by centrifugation, washed once with the dilution buffer, and the bound enzyme eluted with 200 μl of the dilution buffer containing 450 mM

NaCl. The eluted enzyme solution was divided into two unequal
parts, 20% and 80%, and each part was electrophoresed under
non-denaturing conditions into a 6% separating- 4% stacking
native gel system, in adjacent lanes, according to the method

5   of Laemmli (Nature, 227, 680 (1970), except that the SDS is
replaced by 0.2% hydrogenated Triton X-100. Following
electrophoresis, the gel was soaked for ~30 minutes in 0.1 M
sodium acetate, pH 5.5. The "analytical" (20%) and
"preparative" lanes of electrophoresed enzyme (each 7 cm long

10   x 1 cm wide x 1 mm thick) were then cut into ~2.5 mm pieces
using a clean razor blade, sequentially from the top of the
stacker to the bottom of the separating gel. Each of the
analytical slices was combined with 60 μl water and 10 μl 1 M
sodium acetate in a microcentrifuge tube, then homogenized

15   using a Kontes Deltaware motorized pellet pestle
microhomogenizer. MBP-C125SW was added to the desired
concentration (0.5-0.7 ug/ml), and the mixture incubated
overnight. The samples are then diluted with specimen diluent
50-fold, and analyzed by the beta-secretase activity ELISA

20   described below. Preparative slices corresponding to those
analytical slices containing β-secretase activity were then
processed as described below to generate tryptic fragments
from the gel-purified enzyme to obtain partial protein
sequence.

25   Gel slices containing β-secretase activity were
first reduced and alkylated, then digested with trypsin.
Peptides were extracted from the gel pieces and separated by
reverse phase HPLC. Collected purified peptides were
sequenced by automated Edman degradation. Experimental

30   methods follow. Each preparative gel slice was diced into
pieces approximately 1 mm square. To facilitate handling, the
diced pieces of each slice were loaded into individual
microfuge tubes. Pieces were washed twice with 100 μl cold
absolute ethanol per tube. The shrunken pieces were then

35   rehydrated in a volume of Reducing Buffer (0.1M ammonium
bicarbonate, 0.2% hydrogenated Triton X-100, and 0.1M DTT)
sufficient to have about 2 mm of liquid above the pieces,
typically 130-150 μl. The tubes were then incubated at 50°C

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for 30 min with shaking. Following reduction, the pieces were
alkylated by the addition of 25% v/v of 0.5M iodoacetic acid,
usually about 35µl. The tubes were then incubated on ice, in
the dark with shaking, for 45 min. Excess reagents were

5   removed and the pieces partially reshrunken by addition of
sufficient cold absolute ethanol to adjust liquid to 80%
ethanol. The tubes containing the gel pieces and ethanol
solution were then chilled briefly at -20°C, while the trypsin
was prepared.

10   To one vial containing 20 µg of modified, sequencing.
grade trypsin (Promega) was added 50 µl reconstitution buffer
(Promega). After trypsin had completely dissolved, an aliquot
of 12.5 µl was removed and added to 37.5 µl of Digestion
Buffer (0.1M ammonium bicarbonate, 0.2% hydrogenated Triton X-

15   100), yielding a trypsin concentration of 1 µg/10 µl. The
tubes containing the gel pieces were centrifuged briefly, and
the ethanol solutions removed. A further 100 µl of absolute
ethanol was added to each tube to shrink and dehydrate gel
pieces. The tubes were spun and the ethanol removed. To each

20   tube was added about 20 µl of Digestion Buffer in order to
just wet the shrunken gel pieces. Immediately, 10 µl of
prepared trypsin solution was added to each tube. (This
allows the pieces to absorb the trypsin solution completely.)
Sufficient Digestion Buffer was added to leave about 2 mm on

25   top of gel pieces. The tubes were then incubated at 37°C with
gentle shaking for about 2hr. The buffer level was checked
and more Digestion Buffer was added as necessary to maintain
the 2 mm excess. Incubation was allowed to proceed
overnight(~14hr). Tubes were checked and spun briefly to

30   return condensate to the bottoms, and more Digestion Buffer
was added if necessary. After about 20hr, a second aliquot of
freshly prepared trypsin solution (as described above) was
added, and the digestion allowed to proceed for a total of
about 36hr at 37°C.

35   Tubes were taken from 37°C shaker and centrifuged.
The supernatants were removed, combined into a 2ml microfuge
tube and acidifed with trifluoroacetic acid(TFA,double-
distilled). The gel pieces, still in individual tubes, were

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then extracted sequentially with 100μl Digestion Buffer;
100μl 30% acetonitrile(AcN), 0.1% TFA; and 2x 100μl 60%AcN,
0.1% TFA. Each extraction proceeded for 10min at 37°C with
shaking. Extracts were added to combined pool described
5   above. Between extract additions, the pooled volume was
reduced in a Speed-Vac. After the second 60% AcN, 0.1% TFA
extract, the shrunken gel pieces were rehydrated for 1-2min in
about 20μl Digestion Buffer, then immediately extracted for a
third time with 60% AcN, 0.1% TFA. This final extract was
10   combined with the others, and the volume of the pooled
extracts was reduced to about 350μl. TFA (as above) was added
to a final concentration of 1%.

Extracted peptides were loaded onto a Vydac C18
column, 2.1 x 150mm, equilibrated at 40°C in 0.1% TFA, 2% AcN.
15   Purified peptides were eluted with an AcN gradient. Fractions
were collected either by hand or automatically.

Selected peptide fractions were sequenced using an
Applied Biosystems Model 477, equipped with a micro cartridge.
Three unambiguous peptide sequences were obtained, indicated
20   as shown:

#1   AYLTV LGVPE KPQIS GFS(R) [SEQ ID No.:2]
#2   IIPST PFPQE GQPLI LTCE(R) [SEQ ID No.:3]
#3   GKPLP EPVL WTK [SEQ ID No.:4]
25

Synthetic peptides corresponding to part or all of
the above three peptide sequences obtained were generated
using solid-phase peptide synthesis, with the addition of an
amino-terminal linker sequence for two of them (shown
30   underlined), as indicated below:

Peptide Name	Sequence
Seek-1	NH_2CGGYL TVLGV PEKPQ I CONH$_2$
	[SEQ ID No.:5]
Seek-2	AcNHIIP STPFP QEGQP LILTC CO$_2$H
	[SEQ ID No.:6]
Seek-3	NH_2CGGKP LPEPV LWTK CONH$_2$
	[SEQ ID No.:7]

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The synthetic peptides were then conjugated to cationized bovine serum albumin, prior to injection into rabbits for the generation of specific antisera. Antisera obtained at 4 and 10 weeks were then used at 1:500 in a Western blot against partially purified β-secretase. Partially purified β-secretase (Superdex fraction) was electrophoresed into 10-20% Tricine gels under either reducing or non-reducing conditions. Following transfer of the proteins to PVDF membranes, individual lanes are excised, and separately probed with pre-immune serum, and serum obtained at 4 and 10 weeks, from rabbits immunized with the three separate peptide immunogens. The Western blots are developed with secondary donkey-anti rabbit IgG, horseradish peroxidase-linked whole antibody (Amersham), diluted at 1:5000, followed by ECL (Amersham). The results from representative exposures are shown in Fig. 1 (non-reduced) and Fig. 2 (reduced). Individual lanes are marked in both figures. Specific antisera to all three synthetic peptides recognize the same protein band(s) migrating with intermediate mobility between the 60 and 148 kDa molecular weight markers. No immunoreactive bands are detected with pre-immune serum in all cases. The strongest reactivities on Western blot were detected with the specific antisera to Seek-1 (Rabbit 205-A Wk 10) and Seek-3 (Rabbit 211-A Wk 10), with antisera to Seek-2 (Rabbit 210-A Wk 10) producing a much weaker signal. In all cases, much stronger immunoreactivities were evident when the protein was electrophoresed under reducing conditions as compared to non-reducing conditions. This suggests that reducing conditions favor increased exposure of the antigenic epitopes on the otherwise denatured protein, and this was taken into consideration in the design of the immunoprecipitation experiments described below.

A HiTrap Q chromatography fraction containing β-secretase activity was divided in two parts. One of the two aliquots was treated with the reducing agent dithiothreitol (DTT) at 5 mM for 30 min at room temperature. Both the reduced and the untreated, control sample were diluted 10-fold in 20 mM Tris pH 7.5, 2 mM EDTA, 0.2% hydrogenated Triton X-

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100.   Pharmacia Protein A-Sepharose CL-4B was reconstituted by suspending the desiccated resin in the dilution buffer at 75 mg/ml, and letting it stand for 30 min on ice. Enzyme aliquots (100 μl) were then mixed with 20 μl of the

5   reconstituted Protein A-Sepharose and 5 μl of either preimmune rabbit antisera, or antisera from rabbit 205-A Wk 10, or rabbit 211-A Wk 4, and the mixtures incubated for 2 h at room temperature, with gentle end-over-end inversion. Following sedimentation of the Protein A-Sepharose beads (plus bound

10   antibodies and antigens) by microcentrifugation of the incubation mixtures, β-secretase activity was measured in the cleared supernatants using the MBP C125Sw assay. The results are graphically indicated in Fig. 3. The specific antisera against both peptides Seek-1 and Seek-2 immunoprecipitate β-

15   secretase activity under reducing conditions, but not under non-reducing conditions. These results were in agreement with the previous observation that optimal epitope exposure for both antisera require prior reduction of the protein. The immunoprecipitation of β-secretase activity under the

20   conditions of optimal epitope exposure with specific antisera raised to synthetic peptides derived from the non-denaturing gel-purified enzyme confirms that these unique peptide sequences arise from the β-secretase polypeptide.

          In order to generate an amino-terminal protein

25   sequence, it was necessary to alter the native β-secretase by chemical and enzymatic modification to permit separation of minor impurities in the preparation. In order to achieve this, mild reduction, followed by alkylation and partial deglycosylation, was carried out as described below. No loss

30   of enzymatic activity was seen with this sequence of treatments. The LCA-agarose flow-through fraction was concentrated ~5-fold by lyophilization in a SpeedVac vacuum centrifugation apparatus. The reducing agent dithiothreitol (DTT) was added to the enzyme solution to a final

35   concentration of 5 mM, and the preparation incubated at room temperature for 1 h. The carboxamidomethylation reagent iodoacetamide was then added to a final concentration of 10 mM, followed by a further 30 min incubation at room

temperature. The treated enzyme sample was then immediately
desalted using a Pharmacia PD-10 column in order to exchange
the buffer to 20 mM Tris, pH 7.5, 2 mM EDTA, 0.2% hydrogenated
Triton X-100 for the next treatment step.

5   In order to achieve the removal of Asn-linked sugar
chains, 25 mU of the deglycosylating enzyme PNGase F (Glyko)
was added to the β-secretase preparation. This treatment step
was carried out overnight (16 h) at room temperature.

           After β-secretase had thus been reduced,
10   carboxamidomethylated, and (at least partially)
deglycosylated, the treated enzyme was again subjected to
anion-exchange chromatography on a 1 ml HiTrap Q column as
described previously. Eluted fractions containing the peak of
activity were individually concentrated by acetone

15   precipitation, by combining 0.36 ml of each fraction with 1.44
ml of ice-cold acetone, storing the mixture overnight at -40C,
and centrifuging the samples at maximum speed on a benchtop
Eppendorf microcentrifuge for 10 min. After removal of the
supernatant liquid, the pellets were dried down in the

20   SpeedVac vacuum centrifuge apparatus, and the precipitated
protein pellets dissolved in Laemmli sample buffer containing
2% β-mercaptoethanol. The samples were analyzed by
electrophoresis on a Novex 10-20% acrylamide Tricine gel
system, following which the protein bands were visualized

25   using Novex Colloidal Coomassie stain, using the protocol
supplied by the manufacturer. An image of the stained gel was
recorded using a Molecular Dynamics Personal Densitometer. In
order to identify the protein bands corresponding to β-
secretase, a Western blot analysis of a similar gel run in

30   parallel, but with less protein per lane, was carried out
after transfer to PVDF membranes. The Western blot was probed
with antisera against peptide Seek-3 from the previously
described rabbit 211-A wk 10. The results showed that the
triplet of protein bands migrating immediately above the 60

35   kDa MW marker was strongly immunoreactive with this previously
characterized antisera.

           Fractions containing β-secretase activity (#'s 21-
25) were acetone precipitated. Pellets were dried briefly in

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a Speed-Vac to remove residual acetone, dissolved in SDS-PAGE
loading buffer and subjected to SDS-PAGE on a 10-20% Tris-
Tricine gel (Novex). The gel was transferred onto Pro-Blott
membrane (Applied Biosystems) in CAPS buffer. After Coomassie
5   blue staining, three closely spaced bands in the approximate
molecular weight range of 65-75kDa, which coincided in
electrophoretic mobility with the immunoreactivity described
above, were excised for protein sequencing on an Applied
Biosystems Model 477. Two of the bands yielded the same major
10   amino-acid sequence. While the first cycle could not be
identified positively, the next fifteen cycles were called
with reasonable certainty. The consensus sequence obtained
was

15   [S/F/G]KNKVK GSQGQ FPLTQ XVTVV   [SEQ ID No.:8]

β-Secretase Inhibitor Assays
1.   _Assays utilizing purified β-secretase and recombinant_
      fusion peptide substrates
20   β-secretase assays utilizing the SW-192 antibody,
which recognizes the free ...Val-Asn-Leu-COOH terminal
sequence uncovered by proteolytic cleavage immediately amino-
terminal of the βAP sequence, were performed. Two
recombinantly-expressed variants of APP (Figs. 5 and 6) have
25   been used as substrates for β-secretase. Both variants may be
prepared as wild type or Swedish mutations. The preferred
substrate (Fig. 5) was expressed in _E. coli_ as a fusion
protein of the carboxy terminal 125 aa of APP (APP C-125)
fused to the carboxy-terminal end of maltose-binding protein
30   (MBP), using commercially available PMAL vectors from New
England Biolabs. The β-cleavage site was thus 26 amino acids
downstream of the start of the APP C-125 region.
         Recombinant proteins were generated with both the
wild-type APP sequence (MBP-C125 WT) at the cleavage site
35   (..Val-Lys-Met-Asp-Ala..) [SEQ ID No.:9] or the "Swedish"
double mutation (MBP-C125 SW) (..Val-Asn-Leu-Asp-Ala..)
[SEQ ID No.:10]. The entire sequence of the recombinant
protein with the Swedish sequence is given in Fig. 7 [SEQ ID

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No.:1]. As shown in Fig. 4, cleavage of the intact MBP-fusion
protein results in the generation of a truncated amino-
terminal fragment, with the new SW-192 Ab-positive epitope
uncovered at the carboxy terminus. This amino-terminal
5   fragment can be recognized on Western blots with the same Ab,
or, quantitatively, using an anti-MBP capture-biotinylated
SW-192 reporter sandwich format, as shown in Fig. 4.

Anti-MBP polyclonal antibodies were raised in
rabbits (Josman Labs, Berkeley) by immunization with purified
10   recombinantly expressed MBP (New England Biolabs). Antisera
were affinity purified on a column of immobilized MBP.

Fusion peptides comprising the carboxy terminal 125
amino acids of both the Swedish mutation and wild type of APP
(designated MBP-C125 SW and MBP-C125 WT, respectively) were
15   prepared from transfected *E. coli* induced with IPTG,
harvested, and lysed as described in the New England Biolabs
protocol, except that cells were sonicated in lysis buffer
containing 150 mM sodium chloride, 50 mM Tris, pH 7.5, 5 mM
EDTA, and 0.1% Triton X-100. The sonicated cells were
20   pelleted at 10,000 x g for 10 minutes at 4°C and extracted
overnight with 7 M urea, 10 mM Tris, pH 7.5, 5 mM EDTA, and
0.1% Triton X-100. The extract was cleared by centrifugation
at 10,000 x g for 10 minutes, then dialyzed overnight against
lysis buffer. The dialyzed extract was recentrifuged as above
25   and applied to a column of amylose resin (New England
Biolabs). The column was washed extensively (at least 10
column volumes) with lysis buffer, then with two column
volumes of low salt buffer (10 mM Tris, pH 7.5, 5 mM EDTA,
0.1% Triton X-100), and the product was eluted with 10 mM
30   maltose in low salt buffer. The purified substrates were
stored frozen at -40°C in 3 M guanidine-HCl and 0.5 - 0.7%
Triton X-100, at 0.5 - 1.0 mg/ml.

Microtiter 96-well plates were coated with purified
anti-MBP antibody (@ 5-10 μg/ml), followed by blocking with
35   human serum albumin. β-secretase solution (1-10 μl) was mixed
with MBP-C125 SW substrate (0.5 μl) in a final volume of
50 μl, with a final buffer composition of 20 mM sodium
acetate, pH 5.5, 0.035% - 0.05% Triton X-100, in uncoated

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individual wells of 96-well microtiter plates, and incubated
at 37°C for 2 h. For inhibition screening assays, the amount
of β-secretase added was adjusted to give 1600-3200 ng/ml/hr
of product. Samples were then diluted 5-fold with Specimen
5   Diluent (0.2 g/l sodium phosphate monobasic, 2.15 g/l sodium
phosphate dibasic, 0.5 g/l sodium azide, 8.5 g/l sodium
chloride, 0.05% Triton X-405, 6 g/l BSA), further diluted 10-
20 fold into Specimen Diluent on anti-MBP coated plates, and
incubated for 2 h. Biotinylated SW192 antibodies were used as
10   the reporter. SW192 polyclonal antibodies were biotinylated
using NHS-biotin (Pierce), following the manufacturer's
instruction. The biotinylated antibodies were used at about
60-800 ng/ml, the exact concentration was optimized against
MBP-26SW standards (see below) for each lot of antibodies
15   used. Following incubation of the plates with the reporter,
the ELISA was developed using streptavidin-labeled alkaline
phosphatase (Boeringer-Mannheim) and 4-methyl-umbelliferyl
phosphate as fluorescent substrate. Plates were read in a
Cytofluor 2350 Fluorescent Measurement System. Peptides
20   containing maltose-binding protein (MBP) fused to the wild-
type (WT) and Swedish variants (SW) of the βAP sequence (MBP-
26) were prepared as standards by the methods described above
the MBP-C125 substrates, except that the MPB-26 standards were
purified from the lysis buffer in which the E. coli had been
25   sonicated. MBP-26 SW standards were used to generate a
standard curve (Fig. 7), which allowed the conversion of
fluorescent units into amount of product generated.

         This assay protocol was used to screen for inhibitor
structures, using "libraries" of compounds assembled onto 96-
30   well microtiter plates. Compounds were diluted to a stock
concentration of 50 μg/ml in 250 mM sodium acetate, 5% DMSO.
Stocks were centrifuged (1,000 Xg) for five minutes to remove
insoluble compounds, and supernatants added to enzyme and
substrate mixtures to a final concentration of 20 μl/ml DMSO
35   in the assay format described above. The extent of product
generated was compared with control (2% DMSO only) β-secretase
incubations, to calculate "% inhibition." "Hits" were defined
as compounds which result in >35% inhibition of enzyme

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activity at test concentration. Using this system, 14 "hits" were identified out of the first 9944 compounds tested, a "hit" rate of 0.14%. Thus, the assay has been shown to be capable of distinguishing "non-inhibitors" (the majority of

5   compounds) from "inhibitors."

Cleavage by β-secretase of the wild-type MBP-C125 wt was measured by the above procedure, with the following modifications: incubation at 37°C was as above, but in microfuge tubes and for 5 hours. Samples were then diluted 10-

10   fold in Specimen Diluent and transferred to anti-MBP coated plates without further dilution. The reporter antibody was biotinylated wild type specific 192, used at 700 ng/ml. Recombinant wild type MBP-C26 was used to generate a standard curve for conversion of fluorescent units into amount of

15   product (Fig. 9). Varying the amount of β-secretase solution resulted in a corresponding increase in product (Fig. 9), with optimum levels around 6 μl per 50 μl of reaction solution.

2.   Assays Utilizing Partially Purified β-Secretase and
20   Synthetic Oliopeptide Substrates

β-secretase activity was also measured by incubating the partially purified β-secretase preparations with synthetic oligopeptides comprising the cleavage site in APP. The

25   cleavage products could have been detected by any of several techniques, including but not limited to use of fluorescent or chromogenic tags on the N- or C- termini, measurement of free N- or C- termini, or antibody reaction with the cleaved peptides. In the following example, the cleavage products

30   were detected using high performance liquid chromatography (HPLC). The following peptides were employed:

ADRGL TTRPG SGLTN IKTEE ISEVN LDAEF RHDSG YEVHH
QK(26-16'SW) [SEQ ID No.:11]

35

GSGLT NIKTE EISEV NLDAE FRHDS GYEVH HQK(17-16'SW)
[SEQ ID No.:12]

45

ADRGL TTRPG SGLTN IKTEE ISEVN LDAEF(26-4'SW)
[SEQ. ID No.:13]

SEVNL DAEFR HDSGY EVHHQ K(5-16.'SW) [SEQ ID No.:14]

N-AcetylSEVNL DAEFR (5-5'SW) [SEQ ID No.:15]

Peptides were prepared by automated solid phase
synthesis. The 26-4'SW, 5-16'SW, and 5-5'SW peptides were
synthesized with t-BOC chemistry, while the 26-16'SW and 17-
16'SW peptides were synthesized by FMOC chemistry. All
peptides were purified by reverse-phase HPLC before use, using
a 10-50% acetonitrile gradient, at 0.33%/minute, in 0.1% TFA
on a C4 column. The peptide substrate was incubated at 0.3 -
0.35 mg/ml with β-secretase, prepared as described above
through the gel exclusion chromatography step. The β-
secretase-containing gel exclusion fraction was diluted four-
fold in a final assay volume of 250 μl, with a final buffer
composition of 100 mM sodium acetate, pH 5.5, and 0.05%
reduced Triton X-100. The samples are incubated at 37°C for
overnight (18-20 hours) for peptides 26-16'SW, 26-4'SW, or 17-
16'SW, or 70-80 hours for 5-16'SW or 5-5'SW. Following
incubation, trifluoroacetic acid was added to a final
concentration of 1.0 %. The samples were analyzed by HPLC.
26-16'SW, 17-16'SW, or 5-16'SW digests were analyzed on a
Vydac C4 column (4.4 mm x 250 mm, 300 A pore size, 5μ bead
size) using a gradient of 4.5% acetonitrile for 5 minutes,
followed by 4.5-22.5% acetonitrile in 40 minutes, 22.5-31.5%
in 5 minutes, and 31.5-90% in 10 minutes. 26-4'SW and 5-5'SW
digests were analyzed on a Vydac C18 column (4.4 mm x 250 mm,
300 A pore size, 5 μ bead size) using a gradient of 1.8%
acetonitrile for 5 minutes, followed by 1.8-10.8% acetonitrile
in 20 minutes, 10.8-18% in 24 minutes, 18-36% in 36 minutes,
and 36-90% in 15 minutes. Typical separations of digests of
the peptides are shown in Figs. 10A-10E. Product peptides
were identified in selected digests by amino acid analysis and
mass spectroscopy. In addition, the C-terminal Leu/Asp
cleavage product (DAEFRHDSGYEVHHQK) [SEQ ID No.:16] was

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confirmed by comparison with the synthetic peptide. Cleavage
yields were quantitated by measurement of the intensity or
area of the N-terminal (26-4'SW) or C-terminal (all other
peptides) product peptide peak. Standard curves using
5   synthetic DAEFRHDSGYEVHHQK [SEQ ID No.:16] showed that the
HPLC assay was linear and reproducible (Fig. 10F). When
serial gel exclusion fractions from a β-secretase preparation
were analyzed by this method, the results were proportional to
the β-secretase activity as determined by ELISA (Figs. 11A-
10 11C), confirming that the same activity is being measured,
quantitatively, in both assays. The 17-16'SW peptide is the
preferred substrate, since it gave the highest product signal.
Fig. 12 shows the results of assays using the 17-16'SW peptide
and including two candidate inhibitors (Congo Red and an
15 inactive compound which was also inactive by the ELISA assay),
showing that this assay can be used as an alternate screen for
inhibitors, or to verify inhibitors identified in other
assays.

20   3.   Assays Utilizing Transfected Cell Membranes

Generation of β-secretase cleaved APP fragments from
the endogenous full length APP protein was observed using the
192SW antibody (described above) in membranes from 293 cells
25   transfected with the Swedish variant of APP (293SWE cells).
APP fragments may be measured by immunoprecipitation or by
immunoblotting. The latter technique is preferred for reasons
of convenience. Confirmation that the cleavage activity
results from β-secretase was as follows:
30   1) Both activities were highly selective, generating a single
N-terminal product identifiable on immunoblots with the 192SW
antibody (see below).
2) Both activities were membrane-bound.
3) The membrane activity was resistant to the standard
35   protease inhibitors listed in Table 3 above.
A semiquantitative assay for the detecting in situ
β-secretase cleavage of APP in membranes was developed used to
directly identify β-secretase inhibitors. The membrane assay

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is useful as a primary screen or as a secondary assay to
confirm inhibiting activity of potential inhibitors identified
in the assays described above.

5   Cell membrane assays were run as follows. 293SW
cells, were grown in medium containing 90% Dulbecco's MEM, 10%
heat inactivated fetal bovine serum, 25 mM HEPES, 1 mM
pyruvate, 2 mM glutamine, and 0.4 mg/ml geneticin, by standard
procedures (see e.g. R. I. Freshney (1987) Animal Cell
Culture: A Manual of Basic Technique (2nd Edition) Alan R.
10   Liss, Inc. New York, NY). The cells were harvested by rinsing
once with phosphate-buffered saline (PBS), then incubating 5
minutes with PBS containing 2 mM EDTA, with gentle agitation.
All further steps were done at 4°C or on ice, except where
noted. Cells were pelleted by centrifugation for 5 minutes at
15   800 x g, then twice resuspended in PBS and repelleted. The
cell pellet was homogenized in 5 volumes of homogenization
buffer (20 mM HEPES, pH 7.5, 2 mM EDTA, 250 mM sucrose, 1 mM
PMSF, 5 μg/ml dichloroisocoumarin, 1 μg/ml pepstatin A, and 5
μg/ml E-64). The homogenate was centrifuged 10 minutes at 800
20   x g. The supernate was saved, while the pellet was resuspended
in another 5 volumes of homogenization buffer and
recentrifuged. The resulting supernate was pooled with the
previous one, aliquoted into 1.0 ml portions, and respun at
16,000 x g for 20 minutes. The pellets (P2) were stored at -
25   40°C.

Measurement of *in situ* β-secretase activity was
facilitated by extracting the endogenous β-secretase cleaved
APP with saponin. P2 pellets were extracted in 1.0 ml of
resuspension buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 1.0 mM
30   PMSF, 5 μg/ml dichloroisocoumarin, 1 μg/ml pepstatin A, and 5
μg/ml E-64) with 0.02% saponin for 30 minutes, pelleted at
16,000 x g for 20 minutes, then resuspended by vortexing in
the above resuspension buffer, without saponin.

For β-secretase inhibition assays, 10 μl of the test
35   compound of interest at 5 times its desired final
concentration, in 500 mM sodium acetate, pH 5.5, and 20 %
DMSO, was added to 40 μl of the extracted P2 suspension.
Samples were incubated at 37C for 4 hours before stopping the

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reaction with 17 μl of concentrated loading buffer (30%
glycerol, 12% sodium dodecyl sulfate, 400 mM Tris, pH 6.7, 40
mM EDTA, 400 mM dithiothreitol, 0.4 mg/ml Bromophenol Blue).
Samples were boiled and electrophoresed on 10-20% acrylamide
5   Tricine gels (Novex) and transferred to Immobilon membranes
(Millipore). Membranes were blocked in NCS-TBS (10% newborn
calf serum, 150 mM sodium chloride, 50 mM Tris, pH 7.5), and
analyzed using 192SW, 0.5 μg/ml in NCS-TBS, as primary
antibody, horseradish peroxidase-linked anti-rabbit IgG
10   (Amersham) diluted 1:3000 in NCS-TBS as secondary antibody,
and ECL reagent (Amersham) as chemilumenescent developer. The
192SW-reactive bands identified by autoradiography were
quantitated by densitometry.

              As shown in the autoradiograms in Fig. 13, two
15   product bands were identified on immunoblots. The lower and
upper bands correspond to cleavage products of the immature,
core-glycosylated form and the mature, Golgi-processed, fully
glycosylated form of APP, respectively, as shown by their
mobilities on electrophoresis and differential sensitivity to
20   neuraminidase and O-glycanase. As shown by the quantitation,
normalized to the unincubated, unextracted P2 membranes used
as standards (Fig. 14), signal was initially low, and steadily
increased with incubation. Further experiments, such as that
shown in Fig. 15, showed a slight increase in signal with
25   further incubation. Fig. 16 shows the concentration
dependence of inhibition by three putative inhibitors
identified in the ELISA assay. Congo Red and compound 31766
(Fig. 17) were much more potent than an inactive compound in
both the ELISA and the *in situ* assays.

30   Although the foregoing invention has been described
in some detail by way of illustration and example, for
purposes of clarity of understanding, it will be obvious that
certain changes and modifications may be practiced within the
scope of the appended claims.

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SEQUENCE LISTING

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  (ii) TITLE OF INVENTION: Beta-Secretase

 (iii) NUMBER OF SEQUENCES: 17

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      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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      (C) CLASSIFICATION:

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      (A) APPLICATION NUMBER: US 08/480,498
      (B) FILING DATE: 07-JUN-1995

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      (A) APPLICATION NUMBER: US 08/485,152
      (B) FILING DATE: 07-JUN-1995

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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1521 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATGAAAACTG AAGAAGGTAA ACTGGTAATC TGGATTAACG GCGATAAAGG CTATAACGGT    60

CTCGCTGAAG TCGGTAAGAA ATTCGAGAAA GATACCGGAA TTAAAGTCAC CGTTGAGCAT   120

CCGGATAAAC TGGAAGAGAA ATTCCCACAG GTTGCGGCAA CTGGCGATGG CCCTGACATT   180

ATCTTCTGGG CACACGACCG CTTTGGTGGC TACGCTCAAT CTGGCCTGTT GGCTGAAATC   240

ACCCCGGACA AAGCGTTCCA GGACAAGCTG TATCCGTTTA CCTGGGATGC CGTACGTTAC   300

AACGGCAAGC TGATTGCTTA CCCGATCGCT GTTGAAGCGT TATCGCTGAT TTATAACAAA   360

GATCTGCTGC CGAACCCGCC AAAAACCTGG GAAGAGATCC CGGCGCTGGA TAAAGAACTG   420

AAAGCGAAAG GTAAGAGCGC GCTGATGTTC AACCTGCAAG AACCGTACTT CACCTGGCCG   480

CTGATTGCTG CTGACGGGGG TTATGCGTTC AAGTATGAAA ACGGCAAGTA CGACATTAAA   540

GACGTGGGCG TGGATAACGC TGGCGCGAAA GCGGGTCTGA CCTTCCTGGT TGACCTGATT   600

AAAAACAAAC ACATGAATGC AGACACCGAT TACTCCATCG CAGAAGCTGC CTTTAATAAA   660

GGCGAAACAG CGATGACCAT CAACGGCCCG TGGGCATGGT CCAACATCGA CACCAGCAAA   720

GTGAATTATG GTGTAACGGT ACTGCCGACC TTCAAGGGTC AACCATCCAA ACCGTTCGTT   780

GGCGTGCTGA GCGCAGGTAT TAACGCCGCC AGTCCGAACA AGAGCTGGC GAAAGAGTTC   840

CTCGAAAACT ATCTGCTGAC TGATGAAGGT CTGGAAGCGG TTAATAAAGA CAAACCGCTG   900

GGTGCCGTAG CGCTGAAGTC TTACGAGGAA GAGTTGGCGA AGATCCACG TATTGCCGCC   960

ACCATGGAAA ACGCCCAGAA AGGTGAAATC ATGCCGAACA TCCCGCAGAT GTCCGCTTTC  1020

TGGTATGCCG TGCGTACTGC GGTGATCAAC GCCGCCAGCG TCGTCAGAC TGTCGATGAA  1080

GCCCTGAAAG ACGCGCAGAC TAATTCGAGC TCGGTACCCG GCCGGGGATC CATCGAGGGT  1140
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AGGGCCGACC GAGGACTGAC CACTCGACCA GGTTCTGGGT TGACAAATAT CAAGACGGAG   1200

GAGATCTCTG AAGTGAATCT GGATGCAGAA TTCCGACATG ACTCAGGATA TGAAGTTCAT   1260

CATCAAAAAT TGGTGTTCTT TGCAGAAGAT GTGGGTTCAA ACAAAGGTGC AATCATTGGA   1320

CTCATGGTGG GCGGTGTTGT CATAGCGACA GTGATCGTCA TCACCTTGGT GATGCTGAAG   1380

AAGAAACAGT ACACATCCAT TCATCATGGT GTGGTGGAGG TTGACGCCGC TGTCACCCCA   1440

GAGGAGCGCC ACCTGTCCAA GATGCAGCAG AACGGCTACG AAAATCCAAC CTACAAGTTC   1500

TTTGAGCAGA TGCAGAACTA G   1521

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(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Tyr Leu Thr Val Leu Gly Val Pro Glu Lys Pro Gln Ile Ser Gly
1   5   10   15

Phe Ser Arg

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ile Ile Pro Ser Thr Pro Phe Pro Gln Glu Cys Gln Pro Leu Ile Leu
1   5   10   15

Thr Cys Glu Arg
              20

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(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 12 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Lys Pro Leu Pro Glu Pro Val Leu Trp Thr Lys
1   5   10

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (ix) FEATURE:
        (A) NAME/KEY: Region
        (B) LOCATION: one-of(15)
        (D) OTHER INFORMATION: /note= "C-terminal Gln is
            amidated."

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Gly Gly Tyr Leu Thr Val Leu Gly Val Pro Glu Lys Gln Pro
1   5   10   15

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(2) INFORMATION FOR SEQ ID NO:6:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (ix) FEATURE:
          (A) NAME/KEY: Region
          (B) LOCATION: one-of(1)
          (D) OTHER INFORMATION: /note= "N-terminal Asn is
               acetylated."

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

     Asn His Ile Ile Pro Ser Thr Pro Phe Pro Gln Glu Gly Gln Pro Leu
     1   5   10   15

     Ile Leu Thr Cys
             20

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (ix) FEATURE:
        (A) NAME/KEY: Region
        (B) LOCATION: one-of(14)
        (D) OTHER INFORMATION: /note= "C-terminal Lys is
            amidated."

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Cys Gly Gly Lys Pro Leu Pro Glu Pro Val Leu Trp Thr Lys
1   5   10

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(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 21 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
       (A) NAME/KEY: Region
       (B) LOCATION: one-of(1)
       (D) OTHER INFORMATION: /note= "Xaa is Ser, Phe or Gly."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa Lys Asn Lys Val Lys Gly Ser Gln Gly Gln Phe Pro Leu Thr Gln
1   5   10   15

Xaa Val Thr Val Val

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(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val Lys Met Asp Ala
1   5

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(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 5 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Val Asn Leu Asp Ala
1   5

(2) INFORMATION FOR SEQ ID NO:11:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 42 amino acids
          (B) TYPE: amino acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

     Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile
     1   5   10   15

     Lys Thr Glu Glu Ile Ser Glu Val Asn Leu Asp Ala Glu Phe Arg His
             20   25   30

     Asp Ser Gly Tyr Glu Val His His Gln Lys
             35   40

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu Glu Ile Ser Glu Val Asn
1   5   10   15

Leu Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu Val His His Gln
            20   25   30

Lys

(2)   INFORMATION FOR SEQ ID NO:13:

      (i)   SEQUENCE CHARACTERISTICS:
            (A)   LENGTH: 30 amino acids
            (B)   TYPE: amino acid
            (C)   STRANDEDNESS: single
            (D)   TOPOLOGY: linear

      (ii) MOLECULE TYPE: peptide

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ala Asp Arg Gly Leu Thr Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile
1   5   10   15

Lys Thr Glu Glu Ile Ser Glu Val Asn Leu Asp Ala Glu Phe
            20   25   30

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ser Glu Val Asn Leu Asp Ala Glu Phe Arg His Asp Ser Gly Tyr Glu
1   5   10   15

Val His His Gln Lys
            20

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(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Region
        (B) LOCATION: one-of(1)
        (D) OTHER INFORMATION: /note= "N-terminal Ser is acetylated."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ser Glu Val Asn Leu Asp Ala Glu Phe Arg
1   5   10

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(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp Ala Glu Phe Arg His Asp Ser Gln Tyr Glu Val His His Gln Lys
1   5   10   15

(2) INFORMATION FOR SEQ ID NO:17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 506 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

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Met Lys Thr Glu Glu Gly Lys Leu Val Ile Trp Ile Asn Gly Asp Lys
 1               5                  10                  15

Gly Tyr Asn Gly Leu Ala Glu Val Gly Lys Lys Phe Glu Lys Asp Thr
            20                  25                  30

Gly Ile Lys Val Thr Val Glu His Pro Asp Lys Leu Glu Glu Lys Phe
        35                  40                  45

Pro Gln Val Ala Ala Thr Gly Asp Gly Pro Asp Ile Ile Phe Trp Ala
    50                  55                  60

His Asp Arg Phe Gly Gly Tyr Ala Gln Ser Gly Leu Leu Ala Glu Ile
65                  70                  75                  80

Thr Pro Asp Lys Ala Phe Gln Asp Lys Leu Tyr Pro Phe Thr Trp Asp
            85                  90                  95

Ala Val Arg Tyr Asn Gly Lys Leu Ile Ala Tyr Pro Ile Ala Val Glu
            100                 105                 110

Ala Leu Ser Leu Ile Tyr Asn Lys Asp Leu Leu Pro Asn Pro Pro Lys
        115                 120                 125

Thr Trp Glu Glu Ile Pro Ala Leu Asp Lys Glu Leu Lys Ala Lys Gly
    130                 135                 140

Lys Ser Ala Leu Met Phe Asn Leu Gln Glu Pro Tyr Phe Thr Trp Pro
145                 150                 155                 160

Leu Ile Ala Ala Asp Gly Gly Tyr Ala Phe Lys Tyr Glu Asn Gly Lys
                165                 170                 175

Tyr Asp Ile Lys Asp Val Gly Val Asp Asn Ala Gly Ala Lys Ala Gly
            180                 185                 190

Leu Thr Phe Leu Val Asp Leu Ile Lys Asn Lys His Met Asn Ala Asp
        195                 200                 205

Thr Asp Tyr Ser Ile Ala Glu Ala Ala Phe Asn Lys Gly Glu Thr Ala
210                 215                 220
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Met Thr Ile Asn Gly Pro Trp Ala Trp Ser Asn Ile Asp Thr Ser Lys
225             230         235             240

Val Asn Tyr Gly Val Thr Val Leu Pro Thr Phe Lys Gly Gln Pro Ser
                245             250             255

Lys Pro Phe Val Gly Val Leu Ser Ala Gly Ile Asn Ala Ala Ser Pro
            260             265             270

Asn Lys Glu Leu Ala Lys Glu Phe Leu Glu Asn Tyr Leu Leu Thr Asp
        275             280             285

Glu Gly Leu Glu Ala Val Asn Lys Asp Lys Pro Leu Gly Ala Val Ala
    290             295             300

Leu Lys Ser Tyr Glu Glu Glu Leu Ala Lys Asp Pro Arg Ile Ala Ala
305             310             315             320

Thr Met Glu Asn Ala Gln Lys Gly Glu Ile Met Pro Asn Ile Pro Gln
            325             330             335

Met Ser Ala Phe Trp Tyr Ala Val Arg Thr Ala Val Ile Asn Ala Ala
            340             345             350

Ser Gly Arg Gln Thr Val Asp Glu Ala Leu Lys Asp Ala Gln Thr Asn
        355             360             365

Ser Ser Ser Val Pro Gly Arg Gly Ser Ile Glu Gly Arg Ala Asp Arg
    370             375             380

Gly Leu Thr Thr Arg Pro Gly Ser Gly Leu Thr Asn Ile Lys Thr Glu
385             390             395             400

Glu Ile Ser Glu Val Asn Leu Asp Ala Glu Phe Arg His Asp Ser Gly
            405             410             415

Tyr Glu Val His His Gln Lys Leu Val Phe Phe Ala Glu Asp Val Gly
        420             425             430

Ser Asn Lys Gly Ala Ile Ile Gly Leu Met Val Gly Gly Val Val Ile
        435             440             445

Ala Thr Val Ile Val Ile Thr Leu Val Met Leu Lys Lys Lys Gln Tyr
    450             455             460

Thr Ser Ile His His Gly Val Val Glu Val Asp Ala Ala Val Thr Pro
465             470             475             480

Glu Glu Arg His Leu Ser Lys Met Gln Gln Asn Gly Tyr Glu Asn Pro
            485             490             495

Thr Tyr Lys Phe Phe Glu Gln Met Gln Asn
            500             505
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WHAT IS CLAIMED IS:

1   1.   A composition of matter comprising an isolated
2 and purified enzyme which specifically cleaves β-amyloid
3 precursor protein at the β-amyloid peptide cleavage location.

1   2.   A composition of matter comprising β-secretase,
2 wherein the composition has a β-secretase activity which is at
3 least five-fold greater than that of a solubilized but
4 unenriched membrane fraction from human 293 cells.

1   3.   A composition of matter as in claim 2, wherein
2 the β-secretase activity is at least about 100-fold greater
3 than that of a solubilized but unenriched membrane fraction
4 from human 293 cells.

1   4.   A composition of matter comprising at least 10%
2 by weight of an enzyme capable of cleaving β-amyloid precursor
3 protein at the β-amyloid peptide cleavage location and having
4 the following characteristics:
5   (a)   an apparent molecular weight in the range from
6   260 kD to 300 kD measured by gel exclusion
7   chromatography;
8   (b)   a net negative charge at pH 5 and a net
9   negative charge at pH 7.5; and
10   (c)   binds to wheat germ agglutinin with partial
11   binding to other lectins as set forth in Table
12   2.

1   5.   A composition of matter comprising at least 10%
2 by weight of an enzyme capable of cleaving β-amyloid precursor
3 protein at the β-amyloid peptide cleavage location and
4 reactive with antibodies raised against any one of the
5 peptides of [SEQ ID No.:9, SEQ ID No.:10, and SEQ ID No.:11].

1   6.   A composition of matter as in claim 5, wherein
2 the enzyme is reactive with antibodies raised against at least
3 two of the peptides.

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1   7.   A composition of matter as in claim 6, wherein
2   the enzyme is reactive with antibodies raised against all
3   three of the peptides.

1   8.   A composition of matter as in claim 5, wherein
2   the enzyme has an apparent molecular weight between 60 kD and
3   148 kD when determined by electrophoresis.

1   9.   An antibody that specifically binds native β-
2   secretase protein.

1   10.   The antibody of claim 9 that is a polyclonal
2   antibody.

1   11.   The antibody of claim 9 that is a monoclonal
2   antibody.

1   12.   The antibody of claim 9 that is a humanized
2   antibody.

1   13.   The antibody of claim 9 that is an antibody
2   fragment.

1   14.   A method for detecting β-secretase cleavage of
2   a polypeptide substrate, said method comprising:
3   providing a reaction system including β-secretase
4   and the polypeptide substrate present in initial amounts;
5   maintaining the reaction system under conditions
6   which permit β-secretase cleavage of the polypeptide substrate
7   into cleavage products; and
8   detecting the amount of at least one of the β-
1   secretase cleavage products produced as a result of β-
2   secretase cleavage of the substrate.

1   15.   A method as in claim 14, further comprising
2   introducing a test compound to the reaction system and
3   determining whether the test compound affects the amount of β-
4   secretase cleavage product(s) produced.

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1   16.   A method as in claim 14, wherein the β-
2   secretase and the polypeptide substrate are obtained
3   separately and admixed into the reaction mixture.

1   17.   A method as in claim 16, wherein the β-
2   secretase is selected from the group consisting of (1) β-
3   secretase at least partially purified from a cellular source
4   and (2) recombinant β-secretase.

1   18.   A method as in claim 16, wherein the
2   polypeptide substrate is selected from the group consisting of
3   (1) β-amyloid precursor protein (APP) at least partially
4   purified from a cellular source, (2) recombinant polypeptide
5   comprising the β-secretase cleavage site of APP, and (3)
6   synthetic polypeptide comprising the β-secretase cleavage site
7   of APP.

1   19.   A method as in claim 14, wherein the reaction
2   system comprises native β-secretase and native β-amyloid
3   precursor protein (APP) at least partially isolated from a
4   single cellular source.

1   20.   A method as in claim 19, wherein the β-
2   secretase and APP are extracted from cell membranes.

1   21.   A method as in claim 14, wherein the cleavage
2   products include an amino-terminal fragment and a carboxy-
3   terminal fragment and wherein the cleavage product is detected
4   by observing binding between the fragment and a binding
5   substance specific for the carboxy end of the amino-terminal
6   fragment or the amino end of the carboxy-terminal fragment.

1   22.   A method as in claim 19, wherein the generation
2   of the fragment is detected in a gel which separates fragments
3   based upon size and/or electrophoretic mobility.

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1   23. A method for determining whether a test
2   substance inhibits proteolytic cleavage of β-amyloid precursor
3   protein (β-APP), said method comprising:
4   exposing a substrate polypeptide comprising the
5   β-secretase site of APP to an at least partially purified
6   β-secretase polypeptide in the presence of the test substance
7   under conditions such that the β-secretase polypeptide would
8   cleave the polypeptide substrate into an amino-terminal
9   fragment and a carboxy-terminal fragment in the absence of a
10 substance which inhibits such cleavage; and
11   detecting cleavage of the polypeptide at the
12 β-secretase site.

1   24. A method as in claim 23, wherein the at least
2   partially purified β-secretase polypeptide which is in the
3   presence of the substrate polypeptide and test substance has
4   an activity which is at least ten-fold greater than that of a
5   solubilized but unenriched membrane fraction from human 293
6   cells.

1   25. A method as in claim 23, wherein cleavage of
2   the polypeptide is determined by detecting the generation of
3   at least one of the amino-terminal fragment and the carboxy-
4   terminal fragment.

1   26. A method as in claim 25, wherein the generation
2   of the fragment is detected by observing binding between the
3   fragment and a binding substance specific for the carboxy end
4   of the amino-terminal fragment or the amino end of the
5   carboxy-terminal fragment.

1   27. A method as in claim 25, wherein the generation
2   of the fragment is detected in a gel which separates fragments
3   based upon size and/or electrophoretic mobility.

1   28. A method as in claim 25, wherein the
2   polypeptide comprises a sequence including the 125 carboxy-
3   terminal amino acids of β-APP.

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1   29.   A method as in claim 28, wherein the substrate
2   polypeptide is a fusion polypeptide comprising an amino-
3   terminal portion having a binding epitope and a carboxy-
4   terminal portion having the β-secretase site.

1   30.   A method as in claim 29, wherein cleavage of
2   the substrate polypeptide is detected by capture of the amino-
3   terminal portion of the fusion polypeptide and detection of
4   the carboxy end of the amino-terminal portion, wherein said
5   carboxy end is detected by observing binding to a binding
6   substance specific for said carboxy end.

1   31.   A method for determining whether a test
2   substance inhibits proteolytic cleavage of β-amyloid precursor
3   protein (β-APP), said method comprising:
4   exposing a substrate polypeptide comprising the
5   β-secretase site of APP to an at least partially purified
6   β-secretase polypeptide in the presence of the test substance
7   under conditions such that the β-secretase polypeptide would
8   cleave the polypeptide substrate between a Met-Asp or a Leu-
9   Asp cleavage site into an amino-terminal fragment and a
10   carboxy-terminal fragment in the absence of a substance which
11   inhibits such cleavage; and
12   detecting cleavage of the polypeptide at the
13   β-secretase site.

1   32.   A method as in claim 31, wherein the at least
2   partially purified β-secretase polypeptide which is in the
3   presence of the substrate polypeptide and test substance has
4   an activity which is at least five-fold greater than that of a
5   solubilized but unenriched membrane fraction from human 293
6   cells.

1   33.   A method as in claim 31, wherein cleavage of
2   the polypeptide is determined by detecting the generation of
3   at least one of the amino-terminal fragment and the carboxy-
4   terminal fragment.

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1   34.   A method as in claim 33, wherein the generation
2   of the fragment is detected by observing binding between the
3   fragment and a binding substance specific for the carboxy end
4   of the amino-terminal fragment or the amino end of the
5   carboxy-terminal fragment.

1   35.   A method as in claim 33, wherein the generation
2   of the fragment is detected in a gel which separates fragments
3   based upon size and/or electrophoretic mobility.

1   36.   A method as in claim 31, wherein the
2   polypeptide comprises a sequence including the 125 carboxy-
3   terminal amino acids of β-APP.

1   37.   A method as in claim 36, wherein the substrate
2   polypeptide is a fusion polypeptide comprising an amino-
3   terminal portion having a binding epitope and a carboxy-
4   terminal portion having the β-secretase site.

1   38.   A method as in claim 37, wherein cleavage of
2   the substrate polypeptide is detected by capture of the amino-
3   terminal portion of the fusion polypeptide and detection of
4   the carboxy end of the amino-terminal portion, wherein said
5   carboxy end is detected by observing binding to a binding
6   substance specific for said carboxy end.

1   39.   A method as in claim 31, wherein the β-
2   secretase cleaves between amino acid residues 596 and 597 in
3   the 695-isomer of βAPP.

1   40.   A method for inhibiting the cleavage of β-
2   amyloid precursor protein in cells, said method comprising
3   administering to the cells an amount of a compound effective
4   to at least partially inhibit β-secretase activity.

1   41. A method for inhibiting the cleavage of β-
2   amyloid precursor protein in a mammalian host, said method
3   comprising administering to the host an amount of a compound
4   effective to at least partially inhibit β-secretase activity.

1/15
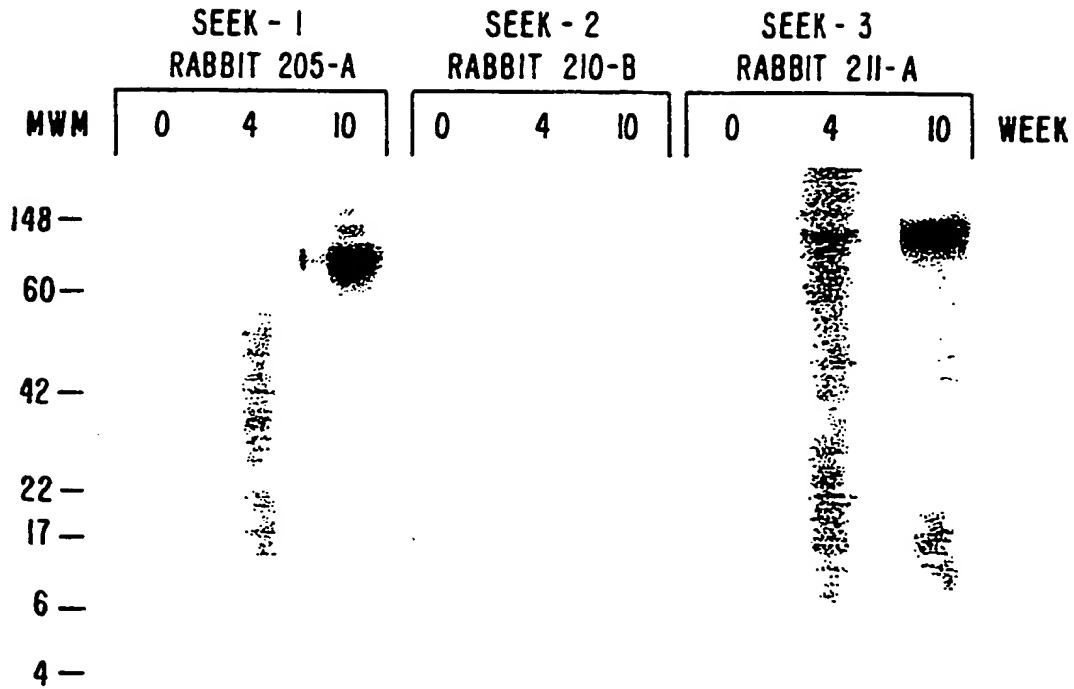
FIG. 1.
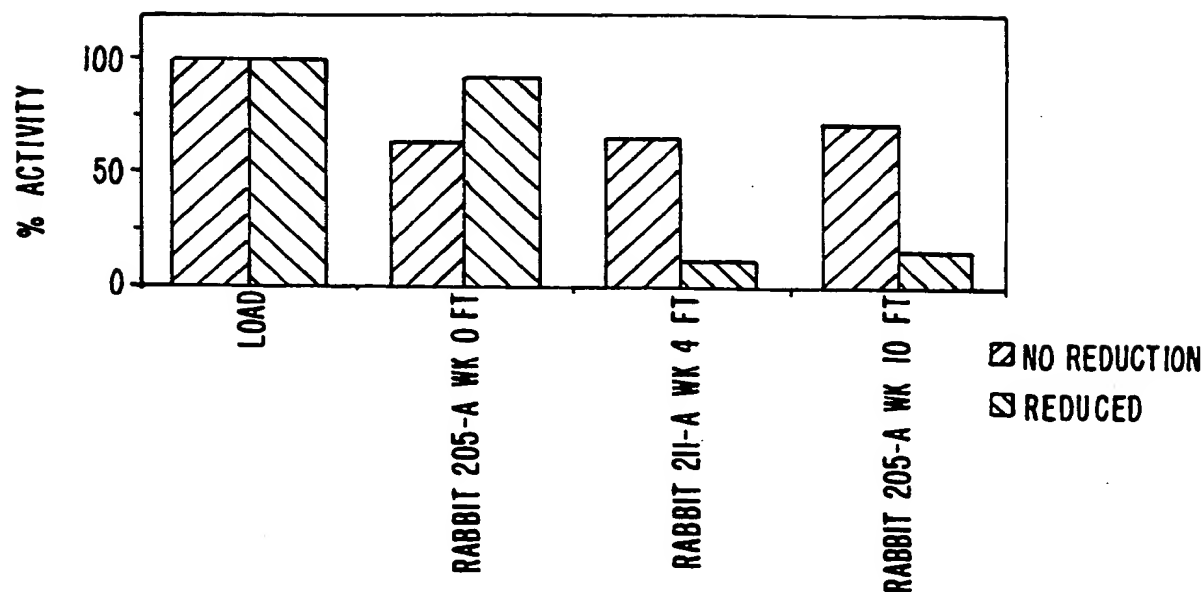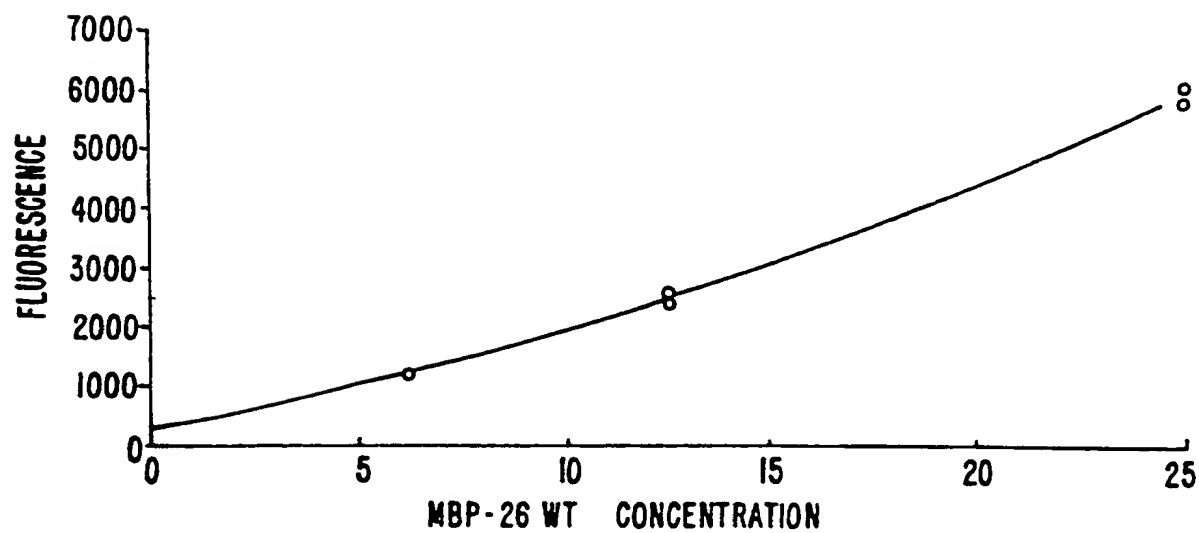
FIG. 2.

FIG. 3.

FIG. 8.

APP C-125

β - SECRETASE

MBP-26   APP C-99

192÷ve

ANTI-MBP CAPTURE   BIOTINYL-SW-192 REPORTER

WILD-TYPE SEQUENCE   ... Val-Lys-Met-Asp
SWEDISH SEQUENCE   ... Val-Asn-Leu-Asp

FIG. 4.

APP 638

β - SECRETASE

8E5   Aβ

192÷ve

DETECTED BY: I. SW 192 WESTERN BLOT
                2. 8E5-192 ELISA

WILD-TYPE SEQUENCE   .... Val-Lys-Met-Asp
SWEDISH SEQUENCE   .... Vab-Asn-Leu-Asp

FIG. 5.

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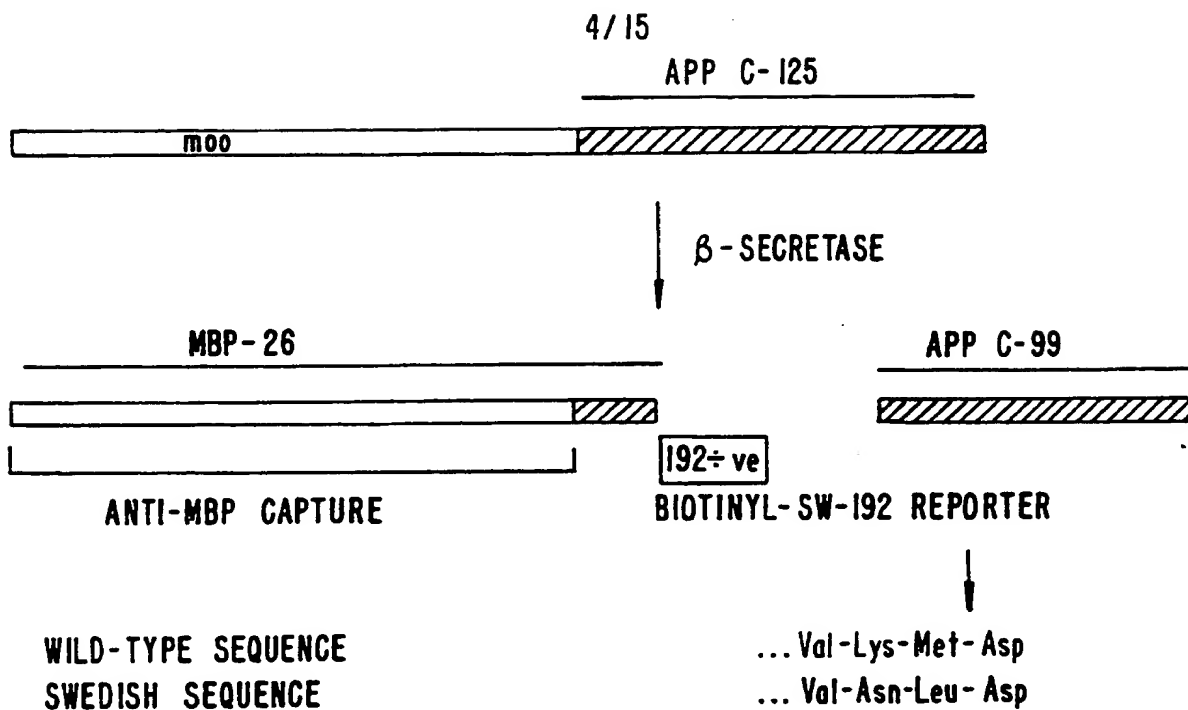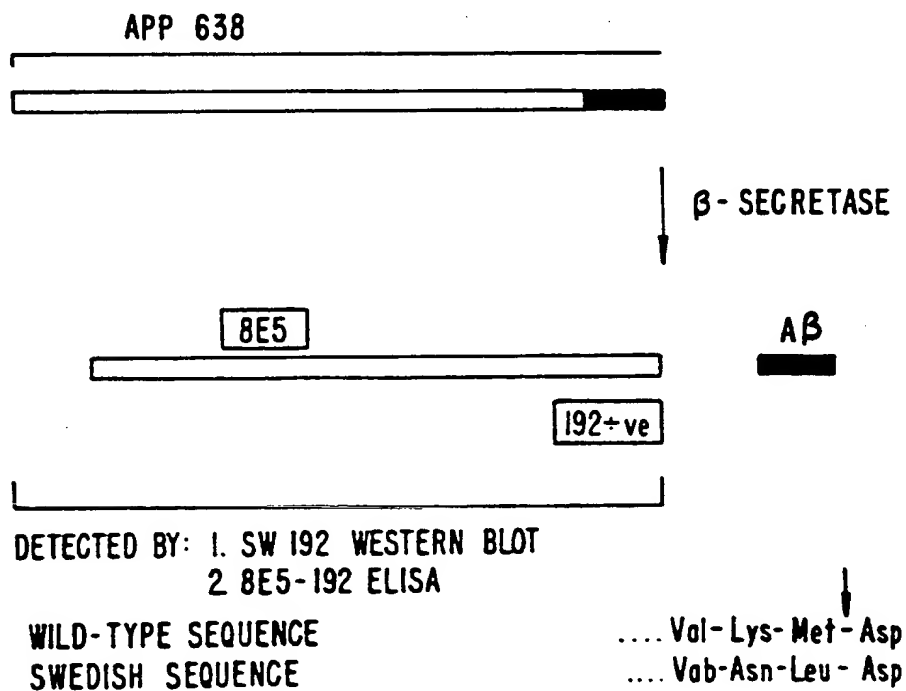
```
  1 ATGAAAACTGAAGAAGGTAAACTGGTAATCTGGATTAACGGCGATAAAGGC
  1 MetLysThrGluGluGlyLysLeuValIleTrpIleAsnGlyAspLysGly

 52 TATAACGGTCTCGCTGAAGTCGGTAAGAAATTCGAGAAAGATACCGGAATT
 18 TyrAsnGlyLewAlaGluValGlyLysLysPheGluLysAspThrGlyIle

103 AAAGTCACCGTTGAGCATCCGGATAAACTGGAAGAGAAATTCCCACAGGTT
 35 LysValThrValGluHisProAspLysLeuGluGluLysPheProGlnVal

154 GCGGCAACTGGCGATGGCCCTGACATTATCTTCTGGGCACACGACCGCTTT
 52 AlaAlaThrGlyAspGlyProAspIleIlePheTrpAlaHisAspArgPhe

205 GGTGGCTACGCTCAATCTGGCCTGTTGGCTGAAATCACCCCGGACAAAGCG
 69 GlyGlyTyrAlaGlnSerGlyLewLewAlaGluIleThrProAspLysAla

256 TTCCAGGACAAGCTGTATCCGTTTACCTGGGATGCCGTACGTTACAACGGC
 86 PheGlnAspLysLeuTyrProTheThrTrpAspAlaValArgTyrAsnGly

307 AAGCTGATTGCTTACCCGATCGCTGTTGAAGCGTTATCGCTGATTTATAAC
103 LysLeuIleAlaTyrProIleAlaValGluAlaLeuSerLeuIleTyrAsn

358 AAAGATCTGCTGCCGAACCCGCCAAAAACCTGGGAAGAGATCCCGGCGCTG
120 LysAspLewLewProAsnProProLysThrTrpGluGluIleProAlaLeu

409 GATAAAGAACTGAAAGCGAAAGGTAAGAGCGCGCTGATGTTCAACCTGCAA
137 AspLysGluLeuLysAlaLysGlyLysSerAlaLeuMetPheAsnLeuGln

460 GAACCGTACTTCACCTGGCCGCTGATTGCTGCTGACGGGGGTTATGCGTTC
154 GluProTyrPheThrTrpProLeuIleAlaAlaAspGlyGlyTyrAlaPhe

511 AAGTATGAAAACGGCAAGTACGACATTAAAGACGTGGGCGTGGATAACGCT
171 LysTyrGluAsnGlyLysTyrAspIleLysAspValGlyValAspAsnAla

562 GGCGCGAAAGCGGGTCTGACCTTCCTGGTTGACCTGATTAAAAACAAACAC
188 GlyAlaLysAlaGlyLewThrPheLeuValAspLeuIleLysAsnLysHis

613 ATGAATGCAGACACCGATTACTCCATCGCAGAAGCTGCCTTTAATAAAGGC
205 MetAsnAlaAspThrAspTyrSerIleAlaGluAlaAlaPheAsnLysGly

664 GAAACAGCGATGACCATCAACGGCCCGTGGGCATGGTCCAACATCGACACC
222 GluThrAlaMetThrIleAsnGlyProTrpAlaTrpSerAsnIleAspThr
```

FIG. 6-1

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```
 715 AGCAAAGTGAATTATGGTGTAACGGTACTGCCGACCTTCAAGGGTCAACCA
 239 SerLysValAsnTyrGlyValThrValLeuProThrPheLysGlyGlnPro

 766 TCCAAACCGTTCGTTGGCGTGCTGAGCGCAGGTATTAACGCCGCCAGTCCG
 256 SerLysProPheValGlyValLeuSerAlaGlyIleAsnAlaAlaSerPro

 817 AACAAAGAGCTGGCGAAAGAGTTCCTCGAAAACTATCTGCTGACTGATGAA
 273 AsnLysGluLeuAlaLysGluPheLeuGluAsnTyrLeuLeuThrAspGlu

 868 GGTCTGGAAGCGGTTAATAAAGACAAACCGCTGGGTGCCGTAGCGCTGAAG
 290 GlyLeuGluAlaValAsnLysAspLysProLeuGlyAlaValAlaLeuLys

 919 TCTTACGAGGAAGAGTTGGCGAAAGATCCACGTATTGCCGCCACCATGGAA
 307 SerTyrGluGluGluLewAlaLysAspProArgIleAlaAlaThrMetGlu

 970 AACGCCCAGAAAGGTGAAATCATGCCGAACATCCCGCAGATGTCCGCTTTC
 324 AsnAlaGlnLysGlyGluIleMetProAsnIleProGlnMetSerAlaPhe

1021 TGGTATGCCGTGCGTACTGCGGTGATCAACGCCGCCAGCGGTCGTCAGACT
 341 TrpTyrAlaValArgThrAlaValIleAsnAlaAlaSerGlyArgGlnThr

1072 GTCGATGAAGCCCTGAAAGACGCGCAGACTAATTCGAGCTCGGTACCCGGC
 358 ValAspGluAlaLeuLysAspAlaGlnThrAsnSerSerSerValProGly

1123 CGGGGATCCATCGAGGGTAGGGCCGACCGAGGACTGACCACTCGACCAGGT
 375 ArgGlySerIleGluGlyArgAlaAspArgGlyLeuThrThrArgProGly

1174 TCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAGTGAATCTGGAT
 392 SerGlyLeuThrAsnIleLysThrGluGluIleSerGluValAsnLeuAsp

1225 GCAGAATTCCGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTG
 409 AlaGluPheArgHisAspSerGlyTyrGluValHisHisGlnLysLeuVal

1276 TTCTTTGCAGAAGATGTGGGTTCAAACAAAGGTGCAATCATTGGACTCATG
 426 PhePheAlaGluAspValGlySerAsnLysGlyAlaIleIleGlyLeuMet

1327 GTGGGCGGTGTTGTCATAGCGACAGTGATCGTCATCACCTTGGTGATGCTG
 443 ValGlyGlyValValIleAlaThrValIleValIleThrLeuValMetLeu

1378 AAGAAGAAACAGTACACATCCATTCATCATGGTGTGGTGGAGGTTGACGCC
 460 LysLysLysGlnTyrThrSerIleHisHisGlyValValGluValAspAla
```

FIG. 6-2

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1429 GCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAACGGCTAC
 477 AlaValThrProGluGluArgHisLeuSerLysMetGlnGlnAsnGlyTyr

1480 GAAAATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAG
 494 GluAsnProThrTyrLysPhePheGluGlnMetGlnAsn...
```

FIG. 6-3

FIG. 7.

FIG. 9.

FIG. IOA.

FIG. IOB.

FIG. 10C.

FIG. 10D.

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FIG. 10E.

FIG. 10F.

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FIG. IIA.

FIG. IIB.

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FIG. IIC.

FIG. 12.

FIG. 13.

SUBSTITUTE SHEET (RULE 26)

FIG. 14.

FIG. 15.

15/15

FIG. 17.

(51) International Patent Classification 6 : **C12N 9/64, C07K 16/18, C12Q 1/37**	**A3**	(11) International Publication Number: **WO 96/40885**
		(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09985

(22) International Filing Date: 7 June 1996 (07.06.96)

(30) Priority Data:
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08/485,152   7 June 1995 (07.06.95)   US

(60) Parent Applications or Grants
  (63) Related by Continuation
  US   08/485,152 (CIP)
  Filed on   7 June 1995 (07.06.95)
  US   08/480,489 (CIP)
  Filed on   7 June 1995 (07.06.95)

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(72) Inventors; and
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(74) Agents: HESLIN, James, M. et al.; Townsend and Townsend and Crew L.L.P., 8th floor, Two Embarcadero Center, San Francisco, CA 94111-3834 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(88) Date of publication of the international search report:
27 February 1997 (27.02.97)

(54) Title: β-SECRETASE, ANTIBODIES TO β-SECRETASE, AND ASSAYS FOR DETECTING β-SECRETASE INHIBITION

(57) Abstract

Compositions comprising a novel protease capable of cleaving β-amyloid precursor protein (APP) on the amino-terminal side of the β-amyloid peptide therein are provided. The protease is designated β-secretase. Reaction systems comprising β-secretase may be used in screening assays to monitor β-secretase modulated cleavage of APP and to identify β-secretase inhibitors, wherein the β-secretase is in the presence of a suitable polypeptide substrate and cleavage of the substrate is determined in the presence and absence of the test substance. Antibodies are raised against peptides of β-secretase. Pharmaceutical compositions and methods comprise compounds identified by screening assays.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N9/64   C07K16/18   C12Q1/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 03542 A (BOSTON UNIVERSITY) 5 March 1992 page 6, first paragraph;pages7-8;page 10, lines 24-28;pages 13-15	1,14-18, 21
Y	page 17, first full paragraph	9-13

X	EP 0 576 152 A (ELI LILLY AND COMPANY) 29 December 1993 Examples 2, 3 and 5; SEQ ID No. 1	1,14-18, 21
Y		9-13

X	WO 92 07068 A (ATHENA NEUROSCIENCES INC.) 30 April 1992 Examples 2, 4 and 7	9-11, 14-18

	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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'A' document defining the general state of the art which is not considered to be of particular relevance

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'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 October 1996	27. 01. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Alt, G

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMISTRY, vol. 33, no. 13, 5 April 1994, pages 3941-3948, XP000605238 MATSUMOTO, A. AND FUJIWARA, Y.: "Ca2+-dependent 68-Kilodalton protease in familial Alzheimer's disease cells cleaves the N-terminus of beta-amyloid" page 3944, left-hand column, last paragraph to page 3946, left-hand column, second paragraph	1,14-22
Y		9-13

X	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 230, 15 May 1995, pages 337-343, XP002015792 MATSUMOTO, A. ET AL.: "Molecular cloning of human cDNA with a sequence highly similar to that of the dihydrofolate reductase gene in brain libraries derived from Alzheimer's disease patients" see page 338, left-hand column, second paragraph	9,10

A	NEURON, vol. 14, March 1995, pages 661-670, XP000602775 CITRON, M. ET AL.: "Generation of amyloid beta protein from its precursor is sequence specific" see the whole document	1,9-22

A	AMYLOID:INTERNATIONAL JOURNAL OF EXPERIMENTAL AND CLINICAL INVESTIGATION, vol. 1, 1 January 1994, pages 263-280, XP000602976 EVIN, G. ET AL.: "Alzheimer's desease amyloid precursor protein (AbetaPP): proteaolytic processing, secretases and betaA4 amyloid production" see pages 272 and 273	1,9-22

P,X	JOURNAL OF NEUROCHEMISTRY, vol. 66, no. 6, June 1996, XP000602767 BROW, A.M. ET AL.: "Evaluation of Cathepsins D and G and EC 3.4.24.15 as candidate beta-secretase proteases using peptide and amyloid precursor protein substrates" see the whole document	1,14-22

1

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

    - claims 1,9-22
    - claims 2,3,23-39
    - claim 4
    - claims 5-8
    - claims 40,41   * see continuation-sheet PCT/ISA/210 *

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

    1,9-22

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/ 210

- claims 1,9-22   : (isolateed and purified) beta-secretase, antibodies
                       directed to it and method using it

- claims 2,3,23-39 : At least partially purified beta-secretase and methods
                     using it

- claim 4   : Beta-secretase having a MW of 260-300 kD, a net negative
                        charge at pH 5 and pH 7,5, and binding to wheat germ
                        agglutinin and other lectics

- claims 5-8   : Beta-secretase being 10% by weight of the composition
                        and reacting with certain antibodies

- claims 40,41   : Method for inhibiting cleavage of beta-amyloid precursor
                        protein

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9203542	05-03-92	AU-B-	643835	25-11-93
		AU-A-	8538791	17-03-92
		CA-A-	2088776	18-02-92
		EP-A-	0546084	16-06-93
		HU-A-	69771	28-09-95
		JP-T-	6503948	12-05-94
		US-A-	5200339	06-04-93
EP-A-0576152	29-12-93	AU-A-	3986493	02-12-93
		BR-A-	9302075	30-11-93
		CA-A-	2096911	29-11-93
		CZ-A-	9300982	16-02-94
		HU-A-	69612	28-09-95
		JP-A-	6062855	08-03-94
		NO-A-	931889	29-11-93
		PL-A-	299053	21-02-94
WO-A-9207068	30-04-92	US-A-	5292652	08-03-94
		US-A-	5424205	13-06-95

(54) Title: PROTEASES CAUSING ABNORMAL DEGRADATION OF AMYLOID β-PROTEIN PRECURSOR

(57) Abstract

A proteolytic factor is capable of cleaving β-protein precursor at a site near the β-protein N-terminus. Also, a method for treating Alzheimer's disease in a patient includes steps of reducing S(b)-protein precursor proteolysis outside the β-protein domain at a site near the β-protein N-terminus. Also, a method for purifying en enzyme from a sample includes steps of incubating the sample with a labelled substrate of the enzyme or with a labelled fragment of a substrate to which the enzyme binds, treating the sample with a crosslinking agent to crosslink any enzyme-substrate complexes in the sample, and recovering labelled complexes. Also, a method for diagnosis in a subject of a disease characterized by accumulation of amyloid includes determining the level, in a sample of tissue or body fluid from the subject, of an AD proteolytic factor. Also, a method for screening for an agent useful in treatment of a disease characterized by accumulation of amyloid includes steps of incubating an AD protease with a peptide having an amino acid sequence corresponding to the sequence spanning the β-protein N-terminus in the presence of a candidate agent, and determining the degradation of the peptide.

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

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DK	Denmark	MC	Monaco		

PROTEASES CAUSING ABNORMAL DEGRADATION
OF AMYLOID β-PROTEIN PRECURSOR

Background of the Invention

This application is a continuation-in-part of my copending application

5   U.S. Serial No. 568,806, filed August 17, 1990.

This invention was made during the course of work supported in part by the U.S. Government, and the Government has certain rights in the invention.

This invention relates to treatment of Alzheimer's disease.

10   The brains of persons having Alzheimer's disease and Down's syndrome and, to a far lesser extent, the brains of normal aged persons exhibit abnormal extracellular proteinaceous deposits, termed amyloid. Amyloid deposits are thought to be trophic or toxic to their surroundings.

Amyloid deposits are found in the center of senile plaques and in the

15   blood vessels in the brains of Alzheimer's disease ("AD") patients. The major component of brain amyloid is the β-protein, a 4 Kd (39-42 amino acids) fragment (see, e.g., G.G. Glenner et al. (1984), Biochem. Biophys. Res. Comm., Vol. 12, pp. 1131-35; C.L. Masters et al. (1985), Proc. Natl. Acad. Sci. USA, Vol. 82, pp. 4245-49; D.J. Selkoe et al. (1986), Jour. Neurochem.,

20   Vol. 46, pp. 1820-34; A. Roher et al. (1986), Proc. Natl. Acad. Sci. USA, Vol. 83, pp. 2662-66, all hereby incorporated herein by reference), derived from a larger, 110-135 Kd β-protein precursor ("β-PP") (see, e.g., D. Goldgaber et al. (1987), Science, Vol. 235, pp. 877080; J. Kang et al. (1987), Nature, Vol. 325, pp. 733-36; N.K. Robakis et al. (19897), Proc. Natl. Acad. Sci. USA, Vol. 84,

25   pp. 4190-94; R.E. Tanzi et al. (1987), Science, Vol. 235, pp. 880-83, all hereby incorporated herein by reference). In addition to and tightly associated with the β-protein, brain amyloid also contains a serine protease inhibitor, α1-antichymotrypsin ("ACT").

Certain β-PP transcripts include a domain homologous to the Kunitz-

30   type protease inhibitors (described, for example, in N. Kitaguchi et al. (1988), Nature, Vol. 331, pp. 530-32; P. Ponte et al. (1988), Nature, Vol. 311,

pp. 525-27; R.E. Tanzi *et al.* (1988), *Nature*, Vol. 331, pp. 528-30). The normal physiologic C-terminal cleavage that releases the secreted form of β-PP (PN2) occurs within the β-protein domain, and outside the putative membrane domain.

5   M. Tsudo *et al.* (1987), *Proc. Natl. Acad. Sci. USA*, Vol. 84, pp. 4215-18) describe crosslinking a ligand and a receptor site for the ligand by treatment with disuccinimidyl suberate ("DSS").

Summary of the Invention

We have discovered proteolytic factors from the brain of AD patients,
10   here termed "AD proteolytic factors". According to the invention accumulation of the β-protein is a consequence of an alternative degradation pathway that results in abnormal β-PP processing, and one or more of the AD proteolytic factors participates in this abnormal pathway.

In general, in one aspect, the invention features an AD proteolytic
15   factor capable of cleaving β-protein precursor at a site near the β-protein N-terminus. In preferred embodiments the AD proteolytic factor is capable of cleaving β-PP at a site outside the β-protein domain and near the β-protein N-terminus, more preferably at a site following lysine or at a site following methionine; a first AD proteolytic factor includes a calcium-
20   activated protease, preferably a serine protease; activity of the serine AD proteolytic factor is inhibited by PN2 and by ACT; a second AD proteolytic factor includes a cysteine protease; the cysteine protease is a metalloprotease, Ca^{2+}- or Mg^{2+}-dependent (and possibly Zn^{2+}-dependent) having a molecular weight about 43-68 kDa.

25   In another general aspect, the invention features a method for treating Alzheimer's disease in a patient, by reducing β-protein precursor proteolysis at a site near the β-protein N-terminus. In preferred embodiments, the method includes administering to the patient an inhibitor that inhibits proteolysis at a site outside the β-protein domain of β-PP and
30   at or near the β-protein N-terminus, and preferably inhibits proteolysis in the vicinity of the β-protein N-terminus, preferably by inhibiting the

proteolytic activity of a proteolytic factor that acts at such a site; the inhibitor is capable of passing the blood-brain barrier, and the inhibitor can be administered, for example, parenterally (intravascularly or intramuscularly) or orally.

5   In another general aspect, the invention features a method for diagnosis in a subject of a disease characterized by accumulation of amyloid, and particularly of Alzheimer's Disease, by determining the level, in a sample from the subject, such as a tissue or fluid sample, of an AD proteolytic factor.

10   In another general aspect, the invention features a method for screening for an agent useful in treatment of a disease characterized by accumulation of amyloid, by incubating an AD protease with a peptide having an amino acid sequence corresponding to the sequence spanning the β-protein N-terminus in the presence of the candidate agent, and

15   determining degradation of the peptide. A candidate agent may be useful in treating such a disease where peptide degradation by the AD protease is less in the presence of the candidate agent than would have been expected under the same or similar reaction conditions in the absence of the candidate agent.

20   In preferred embodiments the peptide has an amino acid sequence that includes a 10-amino acid sequence spanning the β-protein N-terminus, and more preferably beginning five or six amino acids upstream from the N-terminus.

In another general aspect, the invention features a method for

25   purifying an enzyme from a sample, and particularly a proteolytic enzyme, by incubating the sample with a substrate of the enzyme or with a fragment of the substrate to which the enzyme binds, treating the sample with DSS to crosslink any enzyme-substrate complexes in the sample, and recovering the complexes. In preferred embodiments the substrate or substrate

30   fragment is labelled (more preferably radiolabelled).

Description of Pr ferred Embodiments

<u>Drawings</u>

Fig. 1 is a series of prints showing serine protease activity in fractions initially purified from brain homogenates from Alzheimer's disease
5 ("AD") patients. Panel A is a photograph of a coomassie blue stained SDS-PAGE gel showing cleavage products of the iodinated peptide ^{125}I-HSEVKMDAEF (peptide "P1") following reaction with brain homogenates fractions and cross-linking with disuccinimidyl suberate ("DSS"). Panel B is an X-radiograph of a gel prepared as in Panel A. Panel C is an
10 autoradiograph of a cellulose microcrystalline thin layer chromatography ("TLC") plate showing cleavage products of ^{125}I-P1 following reaction with brain homogenate fractions.

Fig. 2 is a series of prints showing serine protease activity in fractions from brain homogenates from AD patients, further purified by size
15 exclusion chromatography. The respective panels A, B, C are as describ d in Fig. 1.

Fig. 3 is a print showing inhibition by various agents of serine protease activity in fractions from brain homogenates from AD patients.

Fig. 4 is a sequence map showing cleavage of P1 by cathepsin G
20 (upper) and by Ca^{2+} activated specific serine protease ("CASP") according to the invention (lower). Abbreviations: H, histidine; S, serine; E, glutamic acid; V, valine; K, lysine; M, methionine; D, aspartic acid; A, alanine; F, phenylalanine. Numerals show percentage cleavage of the peptide bond at each point indicated by an arrow.

25 Fig. 5 is a print of a radiograph of a TLC plate showing cysteine protease activity in fractions purified using DEAE-Trisacryl M ion exchange chromatography from brain homogenates from AD patients. Fractions eluted with a linear NaCl gradient, indicated by the arrow at the lower margin of the Fig., were incubated with radioiodinated P1 and separated on
30 TLC. The sequences of uncleaved P1 and of the cleaved products are shown to the right.

Fig. 6 is a print showing the influenc of dithiothreitol ("DTT") on AD cysteine protease activity. Samples having cysteine AD protease activity were incubated with P1 in the presence of increasing concentrations of DTT and the activity was assayed using TLC. Indicated DTT concentrations: A, DTT solvent alone (no DTT); B, 5.0 mM DTT; C, 2.5 mM DTT; D, 1.0 mM DTT; E, peptide alone (no DTT, no solvent).

Fig. 7 is a print showing inhibition by various agents on cysteine protease activity in fractions from brain homogenates from AD patients.

Isolation and Purification of Cysteine AD proteolytic factor

An AD proteolytic factor according to the invention can be identified in and isolated from a tissue homogenate using, for example, conventional liquid chromatography.

The following is a detailed description, presented by way of example, of a protocol for identifying, isolating, and partially characterizing an AD proteolytic factor from brain homogenate. It will be appreciated that protocols varying in detail from the protocol described here may be used to isolate and purify AD proteolytic factors that are within the scope of the invention.

Generally, the protocol includes steps of homogenizing the tissue; making a crude separation using affinity liquid chromatography; further separating using a first DEAE-ion exchange column, followed by a gel filtration column, followed by a second DEAE-ion exchange column; and dialyzing and finally purifying using affinity liquid chromatography. The description also includes protocols for characterizing the purified AD proteolytic factor (molecular weight; substrate specificity) and for screening for useful inhibitors of the activity.

The protocol described in detail below has been used successfully to isolate and purify a cysteine AD protease from AD brain homogenate. This cysteine AD protease cleaves the P1 peptide after Met. It has a molecular weight about 43-68 kDa, and is a metalloprotein, being Ca^{2+}- or Mg^{2+}-d pendent, and possibly Zn^{2+}-dependent. Apparently, most cysteine

protease inhibitors ffectively inhibit the AD cystein protease purified from AD brain homog nates according to the following protocol.

Brain Homog nates

Brain tissue from AD patients is homogenized in ice-cold 5 x
5   (volume/weight) Tris-Cl buffer containing 1% Triton X-100 and 1 mM dithiothreitol ("DTT") in a Wearing blender. After homogenization, the solution is stirred for 30 minutes on ice, and then centrifuged at 100,000 x g for 60 minutes. The supernatants representing the soluble enzyme are subjected to ammonium sulfate fractionation: 0-25%, 25-50%, 50-75%,
10 >75%, by slowly adding ammonium sulfate salt to the supernatants while stirring on ice. The solution is then stirred for 20 minutes and centrifuged in a Sorval RC-58 refrigerated centrifuge at 10,000 x g for 30 minutes. After the third centrifugation, the three precipitates from the ammonium sulfate fractionation steps are redissolved in Tris-Cl and 1 mM DTT, pH 7.4
15 buffer, and all fractions are dialyzed extensively against the same buffer before further steps.

Synthetic Peptide Substrate

To assay for a protease or proteases that cleave in the vicinity of the N-terminus of the β-protein, an [125]I-labeled peptide having the sequence
20 HSEVKMDAEF (peptide "P1") was synthesized corresponding to the β-PP sequence flanking that site. The peptide starts five amino acids upstream from the N-terminus (the aspartic acid, "D", is at the N-terminus of the β-protein) and extends across the putative cleavage site into the β-protein itself; histidine, "H", was added for purpose of radioiodination (that is,
25 histidine replaces the isoleucine that appears at that site in the native β-protein). Labeled peptide was incubated with brain fractions of varying purity and the resulting fragments were separated by thin layer chromatography ("TLC"); N-terminal fragments were detected by autoradiography. The site of cleavage for an unknown cleavage product is
30 then determined either by direct sequence analysis of the cleavage product, or by comparing the unknown cleavage product with cleavage products

r sulting from a known enzyme such as cathepsin G. Proteolytic activities from AD brain can also be examined by Western blots using full length β-PP derived from rat brain as a substrate.

Assay for Synthetic Peptide Substrate Degrading Activity

5   The different protease fractions are monitored for proteolytic activity against iodinated peptide "P1". Incubations are carried out at 37 °C in 50 mM Tris-Cl, Ph 7.4, in the presence of 1 mM $MgCl_2$ + 1 mM DTT. The proteolytic products are separated by TLC on cellulose microcrystalline plates, using n-butanol:pyridine:acetic acid:water, 15:10:3:12 (v/v), as a

10   solvent, followed by autoradiography.

Purification of Cysteine Protease

Affigel Blue Affinity Chromatography. Affigel Blue is preferred for the first chromatographic step, as it purifies the protease pool from serum albumin and a large number of other protein species. Affigel Blue (Bio-Rad)

15   (1.5 cm x 33 cm) is equilibrated to 50 mM Tris-Cl + 1 mM DTT, pH 7.4, and eluted with a linear gradient of 0 to 0.5 M NaCl. The flow rate is 20 ml/hour, and 3 ml fractions are collected.

First DEAE-Ion Exchange Chromatography. A pool from the Affigel Blue containing the protease activity, as detected using TLC, is loaded on a

20   DEAE-Trisacryl M ion exchange column (1.5 cm x 10 cm). The column is equilibrated with 50 mM Tris-Cl + 1 mM DTT, pH 7.4, and eluted with a linear gradient of 0 to 0.5 M NaCl at a flow rate of 20 ml/hour, and 3 ml fractions are collected.

Gel Filtration Chromatography. A protease activity-positive pool

25   from the first DEAE-Trisacryl M gel filtration step is concentrated to 2.5 ml by ultrafiltration through an Amicon filter (PM-10, 10 kDa cutoff) under nitrogen. The concentrated pool is then loaded on a Sephacryl S-200 gel filtration column (2.5 cm x 66 cm), equilibrated, and eluted with 50 mM Tris-Cl + 1 mM DTT, Ph 7.4. The flow rate is adjusted to 25 ml/hour, and

30   2.2 ml fractions are collected.

- 8 -

Second DEAE-Ion Exchange Chromatography. The pool from the Sephacryl S-200 column containing the protease activity is chromatographed on a second DEAE-Trisacryl M ion exchange column (1.5 cm x 4.5 cm) with a gradient of 0 to 0.5 M NaCl in 50 mM Tris-Cl + 1 mM DTT, pH 7.4. The

5   flow rate is adjusted to 20 ml/h, and 2.2 ml fractions are collected.

Thiopropyl Sepharose 6B Affinity Chromatography. In a final step, the pool from the second DEAE-Trisacryl M gel filtration step containing the protease activity is concentrated to 3 ml by ultrafiltration as before. The concentrated pool is dialyzed overnight in two changes of 500 ml

10   50 mM Tris-Cl, pH 7.4, to remove the DTT. The thiopropyl sepharose 6B resin (0.4 g) is washed with degassed 50 mM Tris-Cl, pH 7.4, followed by 50 mM Tris-Cl + 0.3 M NaCl, pH 7.4, and 2 ml of the following: 50 mM Tris-Cl + 5 mM 2-mercaptoethanol, pH 7.4, and 50 mM Tris-Cl + 10 mM 2-mercaptoethanol, pH 7.4. The flow rate is adjusted to 4 ml/hour, and 2.2 ml

15   fractions are collected.

Molecular Weight Determination

The molecular weight of the protease can be estimated by gel filtration using a Sephacryl S-200 (2.5 cm x 66 cm) column equilibrated with 50 mM Tris-Cl + 1 mM DTT, pH 7.4. The column is eluted with the same

20   buffer at 25 ml/hour, collecting 2.2 ml fractions. The fractions are assayed for proteolytic activity as described above. Protein standards for calibration of the column include β-amylase (200,000 kDa), alcohol dehydrogenase (150,000 kDa), albumin (66,000 kDa), carbonic anhydrase (29,000 kDa), and cytochrome C (12,400 kDa).

25   The apparent molecular weight of the protease can also be determined by SDS-PAGE. Molecular weight standards include: Myosin (H-chain) (228,000 kDa), phosphorylase B (109,600 kDa), bovine serum albumin (70,000 kDa), ovalbumin (44,100 kDa) and carbonic anhydrase (27,900 kDa).

30

Assay for Inhibition of Peptide P1 Degradation by AD Cysteine Protease

To examine the effects of various protease inhibitor reagents on the synthetic peptide P1-degrading activity, the proteolytically active sample is

5   incubated with the appropriate amount of a putative inhibitor reagent for 60 min. at 4 °C, and assayed as described above for remaining proteolytic activity against peptide P1. Control reactions using no inhibitor reagent or containing only the solvent of the reagent are included.

Stock solutions of PMSF are dissolved in 2-propanol, E-64 in

10 dimethylsulfoxide (DMSO), and 1,10 O-phenanthroline and benzamidine-HCl in ethanol. Iodoacetamide, Na-iodoacetate, EGTA, EDTA, bestatin, HMB, and leupeptin are dissolved in double distilled water.

Assay for Substrate Specificity of Purified Cysteine Protease Pool

To examine the substrate specificity of the purified protease pool,

15 aliquots of the purified active fraction were electrophoresed on SDS-substrate gels, containing 1 mg/ml casein or gelatin. The protease pool was mixed on a 1:1 ratio with 2 x Laemmli sample buffer without mercaptoethanol and loaded on a 12% SDS polyacrylamide gel containing 2 x the usual amount of ammonium persulfate. Electrophoresis was carried

20 out at 4 °C at 20 mA. After electrophoresis, the SDS was removed by shaking the gel in 2.5% Triton X-100 for 30 min. at 25 °C. The gel was then incubated in 50 mM Tris-Cl + 1 mM CaCl$_2$ for 2 days at 37 °C while shaking. The gel was stained in 0.5% Coomassie Blue and destained.

Radiolabelling Cysteine AD Protease

25 The proteolytic activity of the purified protease pool made as described above is strongly inhibited by the cysteine protease inhibitor NEM. The protease was labelled with ^{14}C NEM and the sample was analyzed using SDS-PAGE and autoradiography, as follows. Because ^{14}C NEM is provided in n-pentane, the NEM solution was added to an equal

30 volume of double distilled water and the n-pentane was evaporated with a gentle stream of nitrogen gas before use. The protease pool solution was

- 10 -

incubated with ^{14}C NEM (6.7 mM final concentration NEM) at 4 °C for 2
hours. After incubation, the solution was mixed with an equal volume of 2x
sample buffer and then electrophoresed on a 12% SDS acrylamide gel,
generally as described in U.K. Laemmli (1970), *Nature*, Vol. 227, pp. 680-85.

5   Following electrophoresis, the gel was washed in 40% (v/v) methanol, 10%
(v/v) acetic acid for 30 min, and then was washed in Enlightning solution
(New England Nuclear) for 30 min, and then was dried under vacuum and
heat. The gel was then exposed to film for 2 weeks using an intensifying
screen. The cysteine AD protease becomes radiolabelled by virtue of its

10 being bound to the labelled NEM inhibitor.

Characterization of Substrate of Cysteine Protease

The activity of purified AD cysteine protease obtained as described
above was also assayed against a number of chromogenic substrates and full
length β-PP. The protease was incubated with 2 mM substrate solutions of

15 MeOSuc-Glu-Val-Lys-Met-pNA, MeOSuc-Ala-Ala-Pro-Met-pNA, Lys-pNA
and Met-pNA in 50 mM Tris-Cl, 50 mM CaCl$_2$, 100 mM NaCl, 1 mM DTT,
pH 7.9. Changes in absorbance were followed at 410 nm in a Titertek
Multiskan™ ELISA reader. Control reactions contained no enzyme or no
substrate. The activity of the protease was tested against full length β-PP

20 purified from rats by incubating them in 50 mM Tris-Cl, pH 7.4, 1.2 mM
DTT, 1.7 mM MgCl$_2$. The reactions were incubated overnight at 37 °C and
then separated on 7.5% SDS-PAGE gels. The separated polypeptides were
transferred to PDVF membranes (Millipore) generally as described in
Towbin *et al.* (1979), *Proc. Natl. Acad. Sci. USA*, Vol. 76, p. 4350. The blots

25 were immunostained with rabbit anti-β-PP antibody targeted to the area
flanking the N-terminus of the β-peptide, and β-PP fragments were detected
using goat anti-rabbit alkaline phosphatase and the appropriate color
substrate.

Fig. 5 shows cysteine protease activity in fractions purified from brain

30 homogenates from AD patients using DEAE-Trisacryl M ion exchange
chromatography as described above.

Fig. 6 shows the ffect of DTT on AD cysteine protease activity.

Fig. 7 illustrates effects of inhibition by various agents on cysteine protease activity in fractions prepared as described above from brain homogenates from AD patients. The AD cysteine proteolytic factor

5   preparation and the inhibitor (at the indicated concentrations) were incubated at 0 °C for 30 min and then assayed for remaining activity. Lane identifications in Fig. 7 are as follows: lane A, H_2O; lane B, Na iodoacetate, 5 mM; lane C, E-64 (Sigma), 0.02 mM; lane D, E-64, 0.01 mM; lane E, p-hydroxymercuribenzoate, 5 mM; lane F, N-ethylmaleimide, 5 mM; lane G, o-

10   phenanthroline, 4 mM; lane H, o-phenanthroline, 1.8 mM; lane I, ethanol; lane J, ethanol/H_2O; lane K, PMSF, 5 mM; lane L, bestatin, 0.02 mM; lane M, EGTA, 5 mM; lane N, $CaCl_2$, 2 mM; lane O, DTT, 5 mM; lane P, peptide alone.

Isolation and Characterization of Serine AD Proteolytic Factor

15   The following is a detailed description of identification and purification of an AD proteolytic factor that includes a Ca^{2+} activated serine protease whose P1 cleaving activity is inhibited by ACT and PN2.

Brain fractions were incubated with the iodinated peptide (^{125}I-P1) and treated with disuccinimidyl suberate ("DSS") to crosslink any proteins

20   that were in intimate contact with the peptide, i.e., to crosslink any nzyme-substrate complex (and, in this instance, any protease-substrate complexes. Then, the enzymes were recovered in fractions containing a labelled enzyme-substrate complex, stabilized by the DSS crosslinking, and N-terminal fragments were detected by autoradiography on TLC plates,

25   generally as described above in the detailed protocol for the cysteine AD protease.

Employing these assays (TLC, DSS crosslinking), a specific serine protease activity was partially purified from Alzheimer's brain homogenates by classical liquid chromatography.

30   Results of an initial purification of specific protease activity from Alzheimer's disease brain ("AD brain") are shown in Fig. 1. Brain

homog nates were prepared in phosphate buffered saline ("PBS") (20 mM phosphate buffer pH 7.0, 0.15 M NaCl) and 1 mM dithiothreitol ("DDT"), and spun at 10,000 g. The supernatant was dialyzed against PBS (20 mM phosphate buffer pH 7.0, 20 mM NaCl) and 1 mM DDT, and applied to

5 DE52-cellulose (Whatman) equilibrated with 10 mM Tris-HCl (pH 7.0, 20 mM NaCl) and 1 mM DDT; the column was extensively washed and bound proteins were eluted with 0.5 M NaCl. The active fraction was further purified by ammonium sulfate precipitation, followed by dialysis.

The purification was monitored by a peptide degradation assay as

10 follows. All fractions were incubated with iodinated peptide 1 ("^{125}I-P1", amino acid sequence HSEVKMDAEF) in 10 mM Tris-HCl (pH 7.6, 1 mM CaCl$_2$) for one hour, and then the cleavage products were separated by TLC on cellulose microcrystalline plates (J.T. Baker), followed by autoradiography (panel C). The TLC solvent was n-butanol:pyridine:acetic

15 acid:water (15:10:3:12 by volume) as described generally in P. Tempst *et al.* (1983), *Eur. Jour. Biochem.*, Vol. 135, pp. 321-330. Fractions were also reacted with ^{125}I-P1 for 30 minutes at 4 °C, cross-linked with 0.5 mM DSS for 15 minutes at room temperature, and subjected to SDS-PAGE, and the gel was stained with coomassie blue (panel A), dried and exposed to X-ray

20 film (panel B).

Lane identifications in Fig. 1 are as follows: lane 1, Mr standards; lanes 2, 6, 15, DE52 column flowthrough fraction; lanes 3, 7, 10, ammonium sulfate ("AS") precipitation, 0-25% saturation; lanes 4, 8, 11, 25-50% AS; lanes 5, 9, 12, 50-75% AS; lane 13, .75% AS; lane 14, untreated ^{125}I-P1.

25 The asterisk (*) indicates the minor 30 Kd band. Further purification, including a 100,000 g spin in PBS followed by solubilization of the pellet in 1% Triton X-100 in PBS and a second spin at 100,000 g, revealed that following these treatments the enzymatic activity is found in both the soluble fraction and the membrane-bound fraction.

30 Results of purification of a specific serine protease from AD brain by size exclusion chromatography are shown in Fig. 2. An ammonium sulfate

fraction 50-75%, obtained as described above, was dialyzed, applied to a mono Q column (Pharmacia) and eluted with a 20-500 mM NaCl gradient in 10 mM Tris HCl, pH 7.0, with 1 mM DDT. Fractions demonstrating activity were concentrated and applied to a Sephadex S-300 column

5   (Pharmacia) and eluted with 10 mM Tris-HCl, pH 7.0, 200 mM NaCl, and 1 mM DDT.

Panels A, B and C in Fig. 2 are as in Fig. 1. Analysis of S-300 column fractions 14 to 32 is shown; fraction numbers are indicated. The first lane in Panel C is untreated ^{125}I-P1; the second lane is fraction AS 50-

10   75% (before S-300 separation). Fractions 23-28 were reserved for further analysis.

A single protein was radioaffinity labeled (Fig. 1) following initial purification. Subsequent steps of purification resulted in a major protein of approximately 68 Kd (Figs. 1 and 2) and a minor one at 30 Kd (Fig. 1).

15   Fig. 3 illustrates effects of inhibitors on the protease activity of the serine AD proteolytic factor isolated as described above. A peptide degradation assay, using 0.1 μg ^{125}I-P1 for each trial as described above with reference to Fig. 1, was used. Lane identifications in Fig. 3 are as follows: lanes 1 and 11, no protease; lanes 10 and 12, the protease fraction

20   alone ("PF"); lanes 2-9 and 13-16, the protease fraction was treated for 15 minutes at room temperature with various agents, then the P1 was added and the mixture incubated at 37 °C for 1 hour (except lane 9, which was incubated for 20 hours); lane 2, PF with PN1, 1 μM; lane 3, PF with ACT, 0.4 μM; lane 4, PF with ACT, 1 μM; lane 5, PF with ACT, 1.5 μM; lane 6,

25   PF with PN2, 0.2 μM; lane 7, PF with PN2, 0.4 μM; lane 8, PF with PN2, 0.75 μM, for 1 hour; lane 9, PF with PN2, 0.75 μM, for 20 hours; lane 13, PF with β-mercaptoethanol, 0.5 mM; lane 14, PF with 2 mM Ca^{2+} ; lane 15, PF with 2 mM EGTA; lane 16, PF with 1 mM DFP.

EGTA, an inhibitor of Ca^{2+} dependent proteases, and DFP, an

30   inhibitor of serine proteases, prevent cleavage of the ^{125}I-P1 by the AD proteolytic factor (Fig.3), indicating that the fraction was nriched in a Ca^{2+}

activated, serine protease. Additional Ca^{2+} enhances the degradation
(Fig. 3, lane 14). In addition, two serine protease inhibitory proteins from
human brain that may be involved in regulating the degradation of β-PP,
ACT (Calbiochem) and purified PN2 (secreted β-PP), also inhibited cleavage
5   of P1 by the AD proteolytic factor. The complex of the AD proteolytic factor
protease with PN2 is reversible; compare lane 8 (1 hr incubation) with lane
9 (20 hrs incubation). In contrast, protease nexin 1 and albumin did not
influence the activity of the AD proteolytic factor.

The Kunitz-type protease inhibitors are identical to the inhibitor
10   protease nexin 2 ("PN2", described, for example, in W.E. Van Nostrand *et al.*
(1989), *Nature*, Vol. 341, pp. 546-49; T. Oltersdorf *et al.* (1989), *Nature*, Vol.
341, pp. 144-47.

Sequences of the peptide 1 cleavage products generated by
cathepsin G and some other common proteases were compared to those
15   generated by the serine AD protease fraction; the results of a comparison of
P1 cleavage products for Ca^{2+} activated specific serine protease ("CASP")
from Alzheimer's disease brain and cathepsin G on peptide 1 are shown in
Fig. 4. Five micrograms of unlabeled peptide was incubated with the
enzymes in 10 mM Tris-HCl, pH 7.6, 1 mM $CaCl_2$ for 15 hours at 37 °C.
20   Resulting mixtures were analyzed by direct peptide sequencing as described
generally in P. Tempst *et al.* (1989), *Anal. Biochem.*, Vol. 183, pp. 290-300;
the percentage cleavage of each peptide bond is indicated under the arrows.
Unseparated proteolytic fragments were directly sequenced as a mixture.
Of the enzymes tested, only cathepsin G and the AD proteolytic fraction
25   cleaved P1 before and after the methionine, and thus only they among these
enzymes are capable of generating the cleavage to release the N-terminus of
the β-protein from the β-protein precursor. Although cleavage kinetics of
the AD proteolytic factor are relatively slow, a presence of active AD
proteolytic factor in brain would ensure accumulation of the β-protein over
30   time. The partially purified AD protease cleaves the Asp-Ala bond (Fig. 4)
which would result in a β-protein missing the N-terminal Methionine.

Cathepsin G is not detectable in human brain tissue using
immunohistochemistry or Western or northern blot analysis.

The calcium-activated AD proteolytic factor cleaves metabolically
labelled endogenous β-PP substrate as well as the P1 peptide. Labelled ^{35}S-

5   β-PP, made as described in S. Sisodia et al. (1990), Science, Vol. 248, pp.
492-95 and obtained from S. Sisodia, was incubated with the AD proteolytic
factor, and the fragments were separated on gel and the gel was
autoradiographed. A mixture of proteases appear to be present, and one or
more than one of these may play a role in an alternative pathway in the

10   brains of persons having abnormal amyloid deposits.

In addition to being able to degrade the synthetic P1, the serine
protease fraction also degraded purified human PN2, and metabolically
labeled ^{35}Met-PN2 secreted into the medium by cells transfected with the
human β-PP770. Other labeled secreted proteins were not affected by the

15   protease.

Thus far, two protease inhibitors have been described that may be
involved in amyloid deposits of the β-protein type: β-PP (PN2) (the β-
protein precursor) and ACT. In the brain, the two forms of the β-PP, one
having the protease inhibitory domain (751/770 amino acids) and the other

20   lacking it (695 amino acids), are found in comparable amounts, in contrast
to the β-PP in other organs where the inhibitor form prevails. The different
ratios of the two forms may explain the almost unique accumulation of the
β-protein type amyloid in the brain, although β-protein antibodies can also
label skin, intestine and adrenal sections. When the amounts of the β-PP

25   are compared between AD and controls they do not seem significantly
different, but high levels of abnormal degradative forms of β-PP are found
in AD neurons and neurites and on Western blots using β-PP antibodies.

Use

In AD, and most likely in normal aging, the β-protein is probably

30   formed as a result of an abnormal proteolytic degradation of a normal
protein. The finding that the AD cysteine protease cleaves after lysine

suggests that it may also be able to cleave at amino acids 15-17 (QKL; Q,
glutamine; L, lysine) of the β-protein, the normal physiologic site of β-PP
processing. Minor changes in the β-PP, an imbalance in proteases and
inhibitors may influence many normal brain processes, for instance neurite

5   extension.

An aberrant proteolytic degradation of the β-PP can contribute to
amyloid deposition, which in turn may be trophic or toxic to neurons and
astrocytes, causing the neuritic response, neuronal cell death and cognitive
deficits. AD may be treated according to the invention by administering to

10   the patient an inhibitor of the AD proteolytic activity according to the
invention. Such an inhibitor can be, for example, a competitive inhibitor,
such as a fragment of the β-PP molecule corresponding to the binding site of
the proteolytic enzyme.

Likely candidates for effective inhibitors of AD proteolytic activity can

15   be screened by incubating an AD protease according to the invention with a
known specific substrate (such as a synthetic oligopeptide having an amino
acid sequence corresponding to a sequence spanning the β-protein
N-terminus) in the presence of a candidate agent under conditions in which
the AD protease would be expected, in the absence of an inhibitor, to cleave

20   the synthetic oligopeptide near the N-terminus. Candidate agents that
effectively inhibit proteolysis in such a trial can then be tested for inhibitory
effect in an *in vitro* model and/or in an animal model.

A preferred inhibitor is capable of crossing the blood-brain barrier, so
that it can be administered parenterally or orally. Also, a preferred

25   inhibitor is a molecule other than a peptide, so that the inhibitor will not be
rapidly degraded following administration. Also, the preferred inhibitor
specifically inhibits the β-PP cleaving activity of the AD protease, and does
not generally inhibit the activity of brain proteases that are essential to
normal metabolism.

30   Proteolytic fragments resulting from the action on β-PP of an AD
proteas according to the invention can be detected using, *e.g.*, a battery of

antibodies directed against the C-terminus and the N-terminus of the β-PP on Western blots. Such analysis can aid in establishing where the β-PP molecule is cleaved and how the β-protein may be generated. Once the production of the β-protein is carried out *in vitro*, inhibitors (or agents that 5 are candidates for inhibitors) may be screened for capacity to arrest formation of the AD protease *in vitro*.

Monoclonal antibodies ("mAb") raised against a purified AD protease according to the invention, prepared using standard protocols, can be used to establish (and to quantify) the cellular location of the enzyme in the 10 brain and in other tissues such as skin, kidneys, and liver. Such mAb can also be used in the screening of expression libraries, for locating and identifying AD protease-encoding genes, and for cloning such AD protease genes for use in AD protease-producing expression systems.

The damage to tissues resulting from amyloid deposition in AD 15 appears to be irreversible. The neurological symptoms characteristic of AD appear to result from such tissue damage. For a therapy for AD according to the invention to be effective, it should be employed before amyloid deposition has substantially progressed and before neurological symptoms are manifested.

20 Determining the presence of, or monitoring the quantity of, AD proteases can provide a tool for early diagnosis of incipient AD. Tissue or body fluid samples such as, for example, samples of blood, CSF, saliva, urine, can be drawn and assayed for the presence of AD proteases, as an indication of a likelihood of abnormal β-PP metabolism, producing β protein 25 and, ultimately, causing amyloid deposition in tissues.

Other embodiments are within the following claims. For example, any crosslinking agent other than DSS can be used in the enzyme purification protocol, provided that the agent is capable of forming crosslinks in an enzyme-substrate complex between portions of the enzyme 30 and the substrate where the enzyme and substrate are in near proximity.

Claims

1. A proteolytic factor capable of cleaving β-protein precursor at a site outside the β-protein domain and near the β-protein N-terminus.

2. The proteolytic factor of claim 1, said proteolytic factor being capable of cleaving β-PP at a site at the β-protein N-terminus.

3. The proteolytic factor of claim 2, said proteolytic factor being capable of cleaving β-PP at a site following a lysine residue or a methionine residue near the β-protein N-terminus.

4. The proteolytic factor of claim 1, said proteolytic factor comprising a serine protease.

5. The proteolytic factor of claim 1, said proteolytic factor comprising a cysteine protease.

6. A method for treating Alzheimer's disease in a patient, comprising reducing β-protein precursor proteolysis at a site near the β-protein N-terminus.

7. The method of claim 5, comprising administering to the patient an inhibitor that inhibits proteolysis outside the β-protein domain of β-PP and at a site near the β-protein N-terminus.

8. The method of claim 6, comprising administering to the patient an inhibitor that inhibits proteolysis in the vicinity of the β-protein N-terminus.

9. The method of claim 5 wherein said inhibitor inhibits proteolytic activity of a proteolytic factor that acts outside the β-protein domain of β-PP at a site near the β-protein N-terminus.

10. The method of claim 5 wherein said inhibitor inhibits proteolytic activity of a proteolytic factor that acts at a site in the vicinity of the β-protein N-terminus.

11. The method of claim 6 wherein said inhibitor is capable of passing the blood-brain barrier *in vivo*.

12. The method of claim 6 wher in said inhibitor is administered parenterally or orally.

13. A method for purifying an enzyme from a sample, comprising steps of incubating the sample with a labelled substrate of the enzyme or
5   with a labelled fragment of a substrate to which the enzyme binds, treating the sample with a crosslinking agent to crosslink any enzyme-substrate complexes in the sample, and recovering labelled complexes.

14. A method for diagnosis in a subject of a disease characterized by accumulation of amyloid, comprising determining the level, in a sample of
10   tissue or body fluid from the subject, of an AD proteolytic factor.

15. A method for screening for an agent useful in treatment of a disease characterized by accumulation of amyloid, comprising incubating an AD protease with a peptide having an amino acid sequence corresponding to the sequence spanning the β-protein N-terminus in the presence of a
15   candidate agent, and determining the degradation of the peptide.

FIG. 1

FIG. 2

FIG. 3

Cathepsin G　　　H　S　E　V　K　M　D　A　E　F
　　　　　　　　　　　　　　▲　　　▲　▲　▲
　　　　　　　　　　　　　　3　　　100　46　7

CASP　　　　　　　H　S　E　V　K　M　D　A　E　F
　　　　　　　　　　　▲　　　　▲　▲　▲
　　　　　　　　　　　9　　　　21　18　34
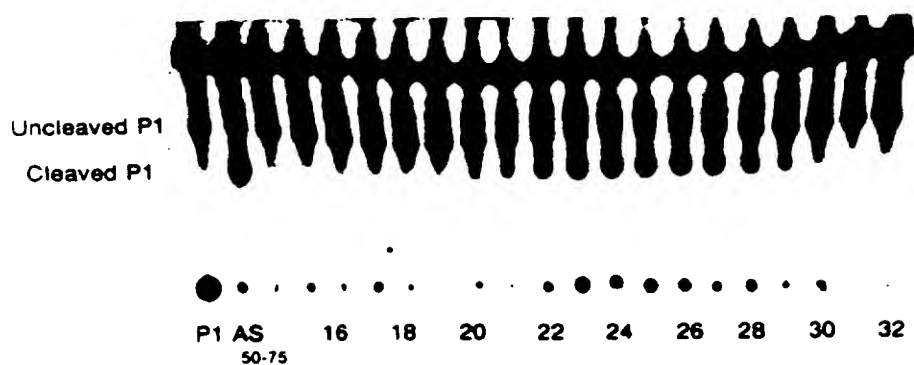
FIG. 4

FIG. 5

FIG. 6

FIG. 7

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 9/50
US CL: 435/219

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US CL:	435/219

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

CAS
BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
X, P	BIOCHEM. BIOPHYS. RES. COMM., Volume 174, No. 2, issued 31 January 1991, Abraham et al., "A Calcium-Activated Protease from Alheimer's Disease Brain Cleaves at the N-Terminus of the Amyloid β-Protein", pages 790-796, see entire document.	1-4
A, P	BIOCHEM. BIOPHYS. RES. COMM., Volume 175, No. 2, issued 15 March 1991, Matsumoto et al., "Abnormal and Deficient Processing of β-Amyloid Precursor Protein in Familiar Alzheimer's Disease Lymphoblastoid Cells", pages 361-365, see summary and introduction.	1-4
A, P	Chemical Abstracts, Volume 114, No. 9, issued 04 March 1991, Siman et al., "Proteolytic Processing of β-Amyloid Precursor by Calpain I", see page 528, column 1, abstract no. 79578, J. Neurosci.. 10(7), 2400-11.	1-4

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 December 1991	20 DEC 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Jon P. Weber

FURTHER INFORMATI N CONTINUED FROM THE SECOND SHEET	
X / Y	J. BIOL. CHEM.. Volume 265. No. 7. issued 05 March 1990, Nelson et al.. "Clipsin. a Chymotrypsin-Like Protease in Rat Brain Which is Irreversibly Inhibited by α-1-Antichymotrypsin". pages 3836-3843. see entire document.
X / Y	PROC. NATL. ACAD. SCI. USA, Volume 87, issued May 1990, Cataldo et al.. "Enzymatically Active Lysosomal Proteases are Associated With Amyloid Deposits in Alzheimer Brain", pages 3861-3865, see entire document.

Right column entries:
1-4 (over) 1-4

1-4 (over) 1-4

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers   . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☒ Claim numbers 7,9,10 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

Method claims cannot depend from a product claim, and clearly define what what is to be searched. Here, the method appears to add no limitations the product.

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment to
Telephone practice Telephone Memorandum

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-4

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: PROTEASES CAUSING ABNORMAL DEGRADATION OF AMYLOID β-PROTEIN PRECURSOR

(57) Abstract

A proteolytic factor is capable of cleaving β-protein precursor at a site near the β-protein N-terminus. Also, a method for treating Alzheimer's disease in a patient includes steps of reducing $(b)-protein precursor proteolysis outside the β-protein domain at a site near the β-protein N-terminus. Also, a method for purifying en enzyme from a sample includes steps of incubating the sample with a labelled substrate of the enzyme or with a labelled fragment of a substrate to which the enzyme binds, treating the sample with a crosslinking agent to crosslink any enzyme-substrate complexes in the sample, and recovering labelled complexes. Also, a method for diagnosis in a subject of a disease characterized by accumulation of amyloid includes determining the level, in a sample of tissue or body fluid from the subject, of an AD proteolytic factor. Also, a method for screening for an agent useful in treatment of a disease characterized by accumulation of amyloid includes steps of incubating an AD protease with a peptide having an amino acid sequence corresponding to the sequence spanning the β-protein N-terminus in the presence of a candidate agent, and determining the degradation of the peptide.

+ DESIGNATIONS OF "SU"

Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

FOR THE PURPOSES OF INFORMATION ONLY

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DK	Denmark	MC	Monaco		

PROTEASES CAUSING ABNORMAL DEGRADATION
OF AMYLOID β-PROTEIN PRECURSOR

Background of the Invention

This application is a continuation-in-part of my copending application

5   U.S. Serial No. 568,806, filed August 17, 1990.

This invention was made during the course of work supported in part
by the U.S. Government, and the Government has certain rights in the
invention.

This invention relates to treatment of Alzheimer's disease.

10   The brains of persons having Alzheimer's disease and Down's
syndrome and, to a far lesser extent, the brains of normal aged persons
exhibit abnormal extracellular proteinaceous deposits, termed amyloid.
Amyloid deposits are thought to be trophic or toxic to their surroundings.

Amyloid deposits are found in the center of senile plaques and in th

15   blood vessels in the brains of Alzheimer's disease ("AD") patients. The
major component of brain amyloid is the β-protein, a 4 Kd (39-42 amino
acids) fragment (see, e.g., G.G. Glenner et al. (1984), Biochem. Biophys. Res.
Comm., Vol. 12, pp. 1131-35; C.L. Masters et al. (1985), Proc. Natl. Acad.
Sci. USA, Vol. 82, pp. 4245-49; D.J. Selkoe et al. (1986), Jour. Neurochem.,

20   Vol. 46, pp. 1820-34; A. Roher et al. (1986), Proc. Natl. Acad. Sci. USA, V 1.
83, pp. 2662-66, all hereby incorporated herein by reference), derived from a
larger, 110-135 Kd β-protein precursor ("β-PP") (see, e.g., D. Goldgaber et al.
(1987), Science, Vol. 235, pp. 877080; J. Kang et al. (1987), Nature, V 1. 325,
pp. 733-36; N.K. Robakis et al. (19897), Proc. Natl. Acad. Sci. USA, Vol. 84,

25   pp. 4190-94; R.E. Tanzi et al. (1987), Science, Vol. 235, pp. 880-83, all
hereby incorporated herein by reference). In addition to and tightly
associated with the β-protein, brain amyloid also contains a serine protease
inhibitor, α1-antichymotrypsin ("ACT").

Certain β-PP transcripts include a domain homologous to the Kunitz-

30   type protease inhibitors (described, for example, in N. Kitaguchi et al.
(1988), Nature, Vol. 331, pp. 530-32; P. Ponte et al. (1988), Nature, Vol. 311,

pp. 525-27; R.E. Tanzi *et al.* (1988), *Nature*, Vol. 331, pp. 528-30). Th
normal physiologic C-terminal cl avag that releases th secreted form of β-
PP (PN2) occurs within the β-protein domain, and outside the putative
membrane domain.

5   M. Tsudo *et al.* (1987), *Proc. Natl. Acad. Sci. USA*, Vol. 84, pp. 4215-
18) describe crosslinking a ligand and a receptor site for the ligand by
treatment with disuccinimidyl suberate ("DSS").

Summary of the Invention

We have discovered proteolytic factors from the brain of AD patients,
10   here termed "AD proteolytic factors". According to the invention
accumulation of the β-protein is a consequence of an alternative degradation
pathway that results in abnormal β-PP processing, and one or more of th
AD proteolytic factors participates in this abnormal pathway.

In general, in one aspect, the invention features an AD proteolytic
15   factor capable of cleaving β-protein precursor at a site near the β-protein
N-terminus. In preferred embodiments the AD proteolytic factor is capable
of cleaving β-PP at a site outside the β-protein domain and near the
β-protein N-terminus, more preferably at a site following lysine or at a site
following methionine; a first AD proteolytic factor includes a calcium-
20   activated protease, preferably a serine protease; activity of the serine AD
proteolytic factor is inhibited by PN2 and by ACT; a second AD proteolytic
factor includes a cysteine protease; the cysteine protease is a
metalloprotease, Ca^{2+}- or Mg^{2+}-dependent (and possibly Zn^{2+}-dependent)
having a molecular weight about 43-68 kDa.

25   In another general aspect, the invention features a method for
treating Alzheimer's disease in a patient, by reducing β-protein precursor
proteolysis at a site near the β-protein N-terminus. In preferred
embodiments, the method includes administering to the patient an inhibitor
that inhibits proteolysis at a site outside the β-protein domain of β-PP and
30   at or near the β-protein N-terminus, and preferably inhibits proteolysis in
the vicinity of the β-protein N-terminus, preferably by inhibiting th

proteolytic activity of a proteolytic factor that acts at such a site; the inhibitor is capable of passing the blood-brain barrier, and the inhibitor can be administered, for example, parenterally (intravascularly or intramuscularly) or orally.

5   In another general aspect, the invention features a method for diagnosis in a subject of a disease characterized by accumulation of amyloid, and particularly of Alzheimer's Disease, by determining the level, in a sample from the subject, such as a tissue or fluid sample, of an AD proteolytic factor.

10   In another general aspect, the invention features a method for screening for an agent useful in treatment of a disease characterized by accumulation of amyloid, by incubating an AD protease with a peptid having an amino acid sequence corresponding to the sequence spanning the β-protein N-terminus in the presence of the candidate agent, and

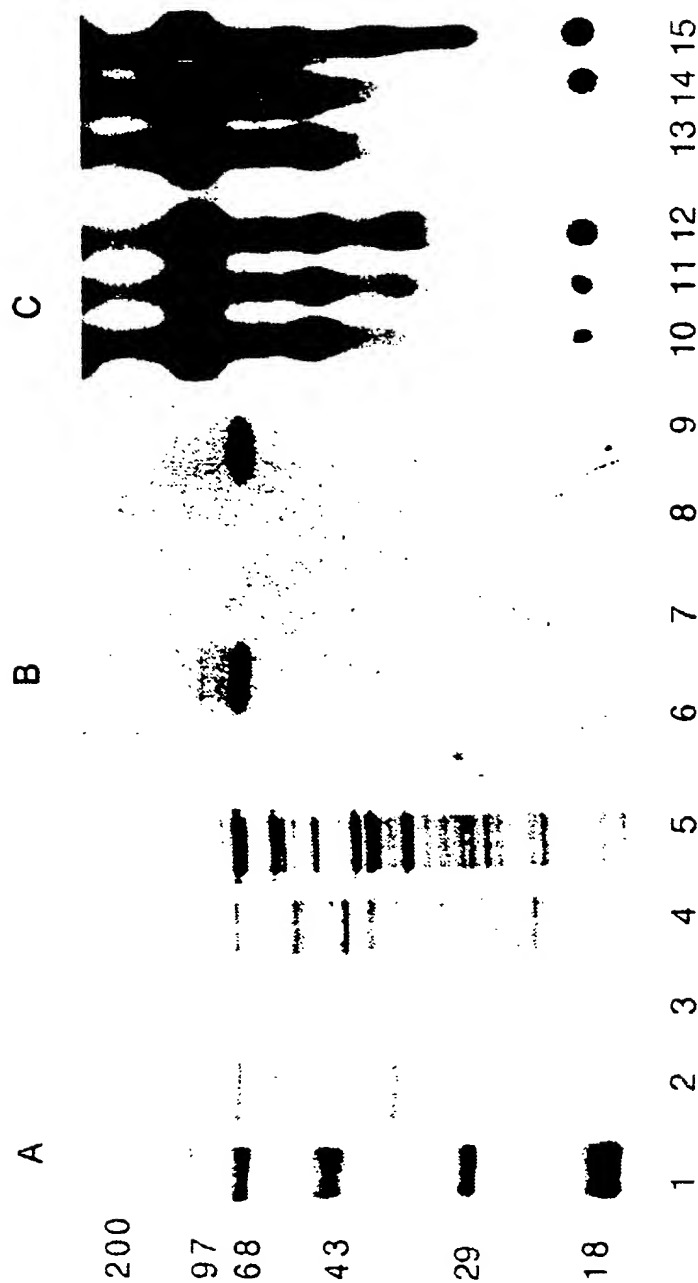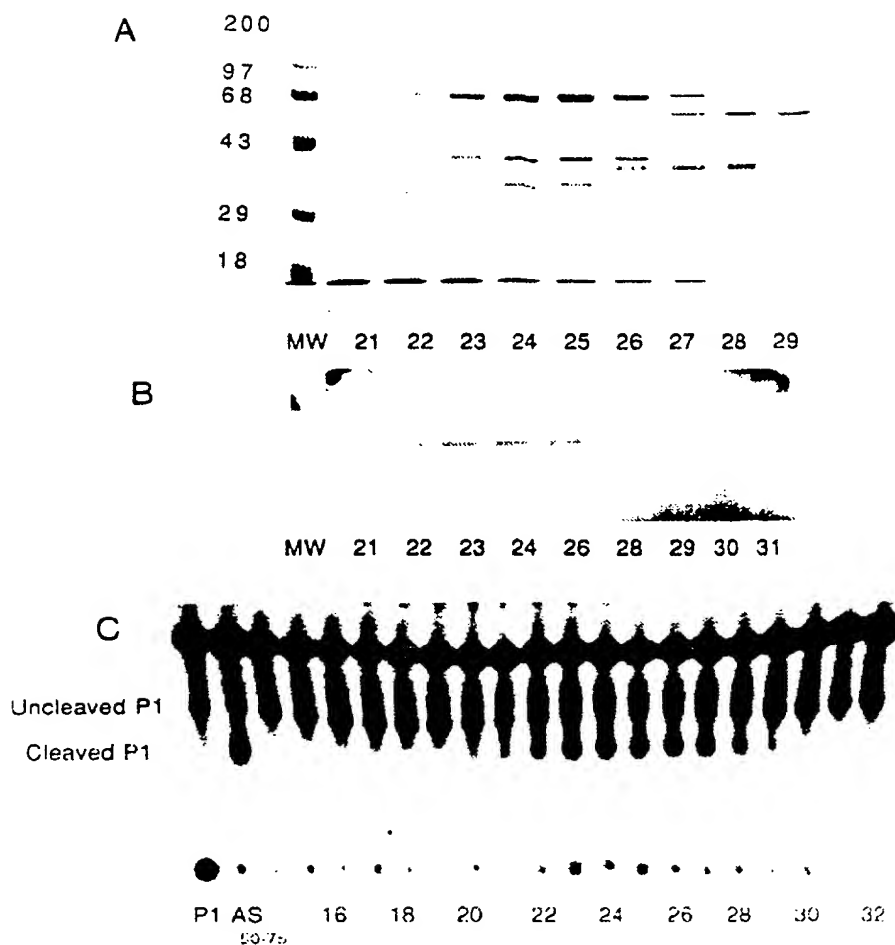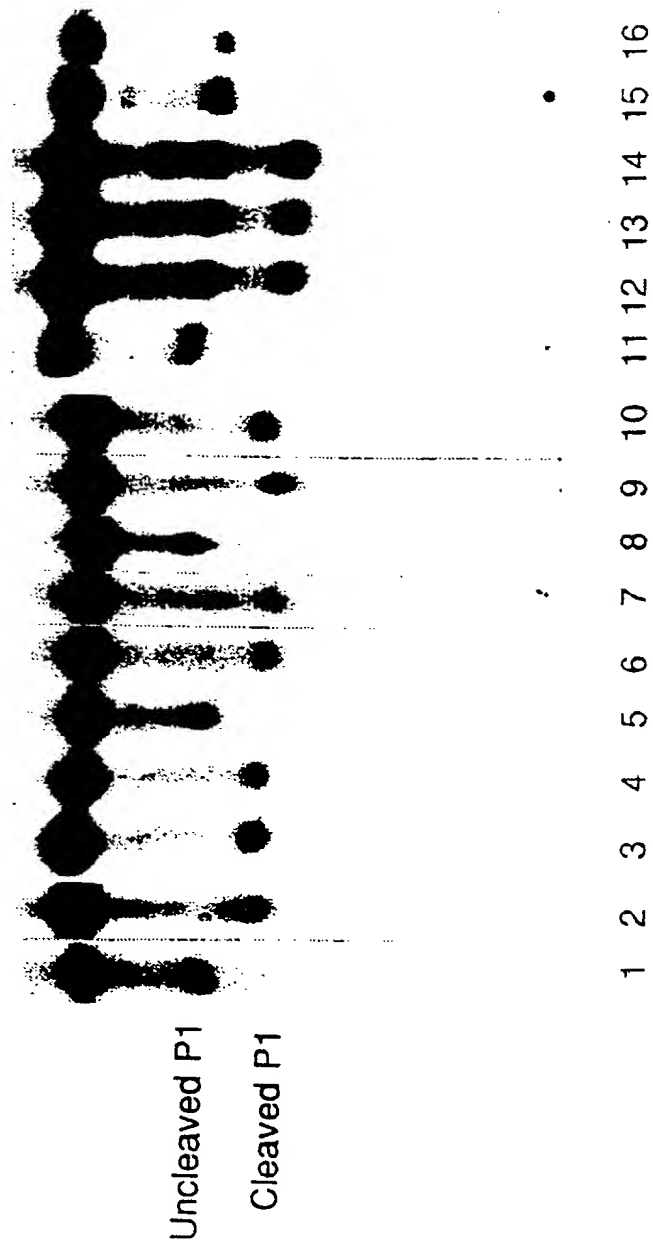15   determining degradation of the peptide. A candidate agent may be useful in treating such a disease where peptide degradation by the AD protease is less in the presence of the candidate agent than would have been expected under the same or similar reaction conditions in the absence of the candidate agent.

20   In preferred embodiments the peptide has an amino acid sequence that includes a 10-amino acid sequence spanning the β-protein N-terminus, and more preferably beginning five or six amino acids upstream from th N-terminus.

In another general aspect, the invention features a method for

25   purifying an enzyme from a sample, and particularly a proteolytic enzyme, by incubating the sample with a substrate of the enzyme or with a fragm nt of the substrate to which the enzyme binds, treating the sample with DSS to crosslink any enzyme-substrate complexes in the sample, and recovering the complexes. In preferred embodiments the substrate or substrate

30   fragment is labell d (more preferably radiolabelled).

Descripti n f Pref rred Embodim nts

<u>Drawings</u>

Fig. 1 is a series of prints showing serine protease activity in fractions initially purified from brain homogenates from Alzheimer's disease

5   ("AD") patients. Panel A is a photograph of a coomassie blue stained SDS-PAGE gel showing cleavage products of the iodinated peptide ^{125}I-HSEVKMDAEF (peptide "P1") following reaction with brain homogenates fractions and cross-linking with disuccinimidyl suberate ("DSS"). Panel B is an X-radiograph of a gel prepared as in Panel A. Panel C is an

10   autoradiograph of a cellulose microcrystalline thin layer chromatography ("TLC") plate showing cleavage products of ^{125}I-P1 following reaction with brain homogenate fractions.

Fig. 2 is a series of prints showing serine protease activity in fractions from brain homogenates from AD patients, further purified by siz

15   exclusion chromatography. The respective panels A, B, C are as described in Fig. 1.

Fig. 3 is a print showing inhibition by various agents of serine protease activity in fractions from brain homogenates from AD patients.

Fig. 4 is a sequence map showing cleavage of P1 by cathepsin G

20   (upper) and by Ca^{2+} activated specific serine protease ("CASP") according to the invention (lower). Abbreviations: H, histidine; S, serine; E, glutamic acid; V, valine; K, lysine; M, methionine; D, aspartic acid; A, alanine; F, phenylalanine. Numerals show percentage cleavage of the peptide bond at each point indicated by an arrow.

25   Fig. 5 is a print of a radiograph of a TLC plate showing cysteine protease activity in fractions purified using DEAE-Trisacryl M ion exchange chromatography from brain homogenates from AD patients. Fractions eluted with a linear NaCl gradient, indicated by the arrow at the lower margin of the Fig., were incubated with radioiodinated P1 and separated on

30   TLC. The sequences of uncl aved P1 and of the cl aved products are shown to the right.

Fig. 6 is a print sh wing th influence of dithi threitol ("DTT") on AD cysteine protease activity. Samples having cystein AD protease activity were incubated with P1 in the presence of increasing concentrations of DTT and the activity was assayed using TLC. Indicated DTT concentrations: A, DTT solvent alone (no DTT); B, 5.0 mM DTT; C, 2.5 mM DTT; D, 1.0 mM DTT; E, peptide alone (no DTT, no solvent).

Fig. 7 is a print showing inhibition by various agents on cysteine protease activity in fractions from brain homogenates from AD patients.

Isolation and Purification of Cysteine AD proteolytic factor

An AD proteolytic factor according to the invention can be identified in and isolated from a tissue homogenate using, for example, conventional liquid chromatography.

The following is a detailed description, presented by way of example, of a protocol for identifying, isolating, and partially characterizing an AD proteolytic factor from brain homogenate. It will be appreciated that protocols varying in detail from the protocol described here may be used to isolate and purify AD proteolytic factors that are within the scope of th invention.

Generally, the protocol includes steps of homogenizing the tissue; making a crude separation using affinity liquid chromatography; further separating using a first DEAE-ion exchange column, followed by a gel filtration column, followed by a second DEAE-ion exchange column; and dialyzing and finally purifying using affinity liquid chromatography. The description also includes protocols for characterizing the purified AD proteolytic factor (molecular weight; substrate specificity) and for screening for useful inhibitors of the activity.

The protocol described in detail below has been used successfully to isolate and purify a cysteine AD protease from AD brain homogenate. This cysteine AD protease cleaves the P1 peptide after Met. It has a molecular weight about 43-68 kDa, and is a metalloprotein, being Ca^{2+}- or Mg^{2+}-dependent, and possibly Zn^{2+}-d pendent. Apparently, most cysteine

protease inhibitors effectively inhibit the AD cystein protease purified from AD brain homogenates acc rding to th following protocol.

Brain Homogenates

Brain tissue from AD patients is homogenized in ice-cold 5 x
5   (volume/weight) Tris-Cl buffer containing 1% Triton X-100 and 1 mM dithiothreitol ("DTT") in a Wearing blender. After homogenization, th solution is stirred for 30 minutes on ice, and then centrifuged at 100,000 x g for 60 minutes. The supernatants representing the soluble enzyme ar subjected to ammonium sulfate fractionation: 0-25%, 25-50%, 50-75%,
10   >75%, by slowly adding ammonium sulfate salt to the supernatants whil stirring on ice. The solution is then stirred for 20 minutes and centrifuged in a Sorval RC-58 refrigerated centrifuge at 10,000 x g for 30 minutes. After the third centrifugation, the three precipitates from the ammonium sulfate fractionation steps are redissolved in Tris-Cl and 1 mM DTT, pH 7.4
15   buffer, and all fractions are dialyzed extensively against the same buffer before further steps.

Synthetic Peptide Substrate

To assay for a protease or proteases that cleave in the vicinity of th N-terminus of the β-protein, an ^{125}I-labeled peptide having the sequence
20   HSEVKMDAEF (peptide "P1") was synthesized corresponding to the β-PP sequence flanking that site. The peptide starts five amino acids upstream from the N-terminus (the aspartic acid, "D", is at the N-terminus of the β-protein) and extends across the putative cleavage site into the β-protein itself; histidine, "H", was added for purpose of radioiodination (that is,
25   histidine replaces the isoleucine that appears at that site in the native β-protein). Labeled peptide was incubated with brain fractions of varying purity and the resulting fragments were separated by thin layer chromatography ("TLC"); N-terminal fragments were detected by autoradiography. The site of cleavage for an unknown cleavage product is
30   th n determined either by direct sequence analysis of th cleavage product, or by comparing th unknown cleavage product with cl avage products

resulting from a known nzyme such as cath psin G. Proteolytic activities from AD brain can also be examined by Western blots using full length β-PP derived from rat brain as a substrate.

Assay for Synthetic Peptide Substrate Degrading Activity

5   The different protease fractions are monitored for proteolytic activity against iodinated peptide "P1". Incubations are carried out at 37 °C in 50 mM Tris-Cl, Ph 7.4, in the presence of 1 mM $MgCl_2$ + 1 mM DTT. The proteolytic products are separated by TLC on cellulose microcrystalline plates, using n-butanol:pyridine:acetic acid:water, 15:10:3:12 (v/v), as a 10   solvent, followed by autoradiography.

Purification of Cysteine Protease

Affigel Blue Affinity Chromatography. Affigel Blue is preferred for the first chromatographic step, as it purifies the protease pool from serum albumin and a large number of other protein species. Affigel Blue (Bio-Rad) 15   (1.5 cm x 33 cm) is equilibrated to 50 mM Tris-Cl + 1 mM DTT, pH 7.4, and eluted with a linear gradient of 0 to 0.5 M NaCl. The flow rate is 20 ml/hour, and 3 ml fractions are collected.

First DEAE-Ion Exchange Chromatography. A pool from the Affigel Blue containing the protease activity, as detected using TLC, is loaded on a 20   DEAE-Trisacryl M ion exchange column (1.5 cm x 10 cm). The column is equilibrated with 50 mM Tris-Cl + 1 mM DTT, pH 7.4, and eluted with a linear gradient of 0 to 0.5 M NaCl at a flow rate of 20 ml/hour, and 3 ml fractions are collected.

Gel Filtration Chromatography. A protease activity-positive pool 25   from the first DEAE-Trisacryl M gel filtration step is concentrated to 2.5 ml by ultrafiltration through an Amicon filter (PM-10, 10 kDa cutoff) under nitrogen. The concentrated pool is then loaded on a Sephacryl S-200 g 1 filtration column (2.5 cm x 66 cm), equilibrated, and eluted with 50 mM Tris-Cl + 1 mM DTT, Ph 7.4. The flow rate is adjusted to 25 ml/hour, and 30   2.2 ml fractions are collected.

- 8 -

Second DEAE-Ion Exchange Chromatography. The pool from the Sephacryl S-200 column containing the protease activity is chromatographed on a second DEAE-Trisacryl M ion exchange column (1.5 cm x 4.5 cm) with a gradient of 0 to 0.5 M NaCl in 50 mM Tris-Cl + 1 mM DTT, pH 7.4. The

5   flow rate is adjusted to 20 ml/h, and 2.2 ml fractions are collected.

Thiopropyl Sepharose 6B Affinity Chromatography. In a final step, the pool from the second DEAE-Trisacryl M gel filtration step containing the protease activity is concentrated to 3 ml by ultrafiltration as before. The concentrated pool is dialyzed overnight in two changes of 500 ml

10   50 mM Tris-Cl, pH 7.4, to remove the DTT. The thiopropyl sepharose 6B resin (0.4 g) is washed with degassed 50 mM Tris-Cl, pH 7.4, followed by 50 mM Tris-Cl + 0.3 M NaCl, pH 7.4, and 2 ml of the following: 50 mM Tris-Cl + 5 mM 2-mercaptoethanol, pH 7.4, and 50 mM Tris-Cl + 10 mM 2-mercaptoethanol, pH 7.4. The flow rate is adjusted to 4 ml/hour, and 2.2 ml

15   fractions are collected.

Molecular Weight Determination

The molecular weight of the protease can be estimated by gel filtration using a Sephacryl S-200 (2.5 cm x 66 cm) column equilibrated with 50 mM Tris-Cl + 1 mM DTT, pH 7.4. The column is eluted with the same

20   buffer at 25 ml/hour, collecting 2.2 ml fractions. The fractions are assayed for proteolytic activity as described above. Protein standards for calibration of the column include β-amylase (200,000 kDa), alcohol dehydrogenase (150,000 kDa), albumin (66,000 kDa), carbonic anhydrase (29,000 kDa), and cytochrome C (12,400 kDa).

25   The apparent molecular weight of the protease can also be determined by SDS-PAGE. Molecular weight standards include: Myosin (H-chain) (228,000 kDa), phosphorylase B (109,600 kDa), bovine serum albumin (70,000 kDa), ovalbumin (44,100 kDa) and carbonic anhydrase (27,900 kDa).

30

Assay for Inhibition of Peptide P1 Degradation by AD Cysteine Protease

To examine the effects of various protease inhibitor reagents on th synthetic peptide P1-degrading activity, the proteolytically active sampl is

5   incubated with the appropriate amount of a putative inhibitor reagent f r 60 min. at 4 °C, and assayed as described above for remaining proteolytic activity against peptide P1. Control reactions using no inhibitor reagent or containing only the solvent of the reagent are included.

Stock solutions of PMSF are dissolved in 2-propanol, E-64 in

10   dimethylsulfoxide (DMSO), and 1,10 O-phenanthroline and benzamidine-HCl in ethanol. Iodoacetamide, Na-iodoacetate, EGTA, EDTA, bestatin, HMB, and leupeptin are dissolved in double distilled water.

Assay for Substrate Specificity of Purified Cysteine Protease Pool

To examine the substrate specificity of the purified protease pool,

15   aliquots of the purified active fraction were electrophoresed on SDS-substrate gels, containing 1 mg/ml casein or gelatin. The protease pool was mixed on a 1:1 ratio with 2 x Laemmli sample buffer without mercaptoethanol and loaded on a 12% SDS polyacrylamide gel containing 2 x the usual amount of ammonium persulfate. Electrophoresis was carried

20   out at 4 °C at 20 mA. After electrophoresis, the SDS was removed by shaking the gel in 2.5% Triton X-100 for 30 min. at 25 °C. The gel was then incubated in 50 mM Tris-Cl + 1 mM CaCl$_2$ for 2 days at 37 °C while shaking. The gel was stained in 0.5% Coomassie Blue and destained.

Radiolabelling Cysteine AD Protease

25   The proteolytic activity of the purified protease pool made as described above is strongly inhibited by the cysteine protease inhibitor NEM. The protease was labelled with ^{14}C NEM and the sample was analyzed using SDS-PAGE and autoradiography, as follows. Because ^{14}C NEM is provided in n-pentane, the NEM solution was added to an equal

30   volume of double distilled water and th n-pentane was evaporated with a gentle stream of nitrogen gas before use. Th protease pool solution was

- 10 -

incubated with ^{14}C NEM (6.7 mM final concentration NEM) at 4 °C f r 2 hours. After incubation, the solution was mixed with an equal volume of 2x sample buffer and then electrophoresed on a 12% SDS acrylamide gel, generally as described in U.K. Laemmli (1970), *Nature*, Vol. 227, pp. 680-85.

5   Following electrophoresis, the gel was washed in 40% (v/v) methanol, 10% (v/v) acetic acid for 30 min, and then was washed in Enlightning solution (New England Nuclear) for 30 min, and then was dried under vacuum and heat. The gel was then exposed to film for 2 weeks using an intensifying screen. The cysteine AD protease becomes radiolabelled by virtue of its

10   being bound to the labelled NEM inhibitor.

Characterization of Substrate of Cysteine Protease

The activity of purified AD cysteine protease obtained as described above was also assayed against a number of chromogenic substrates and full length β-PP. The protease was incubated with 2 mM substrate solutions of

15   MeOSuc-Glu-Val-Lys-Met-pNA, MeOSuc-Ala-Ala-Pro-Met-pNA, Lys-pNA and Met-pNA in 50 mM Tris-Cl, 50 mM CaCl₂, 100 mM NaCl, 1 mM DTT, pH 7.9. Changes in absorbance were followed at 410 nm in a Titertek Multiskan™ ELISA reader. Control reactions contained no enzyme or no substrate. The activity of the protease was tested against full length β-PP

20   purified from rats by incubating them in 50 mM Tris-Cl, pH 7.4, 1.2 mM DTT, 1.7 mM MgCl₂. The reactions were incubated overnight at 37 °C and then separated on 7.5% SDS-PAGE gels. The separated polypeptides were transferred to PDVF membranes (Millipore) generally as described in Towbin *et al.* (1979), *Proc. Natl. Acad. Sci. USA*, Vol. 76, p. 4350. The blots

25   were immunostained with rabbit anti-β-PP antibody targeted to the area flanking the N-terminus of the β-peptide, and β-PP fragments were detected using goat anti-rabbit alkaline phosphatase and the appropriate color substrate.

Fig. 5 shows cystein protease activity in fractions purified from brain

30   homog nates fr m AD patients using DEAE-Trisacryl M i n exchange chromatography as described above.

Fig. 6 shows th effect of DTT on AD cysteine protease activity.

Fig. 7 illustrates effects of inhibition by various agents on cysteine protease activity in fractions prepared as described above from brain homogenates from AD patients. The AD cysteine proteolytic factor

5 preparation and the inhibitor (at the indicated concentrations) were incubated at 0 °C for 30 min and then assayed for remaining activity. Lane identifications in Fig. 7 are as follows: lane A, H$_2$O; lane B, Na iodoacetate, 5 mM; lane C, E-64 (Sigma), 0.02 mM; lane D, E-64, 0.01 mM; lane E, p-hydroxymercuribenzoate, 5 mM; lane F, N-ethylmaleimide, 5 mM; lan G, o-

10 phenanthroline, 4 mM; lane H, o-phenanthroline, 1.8 mM; lane I, ethanol; lane J, ethanol/H$_2$O; lane K, PMSF, 5 mM; lane L, bestatin, 0.02 mM; lan M, EGTA, 5 mM; lane N, CaCl$_2$, 2 mM; lane O, DTT, 5 mM; lane P, peptide alone.

Isolation and Characterization of Serine AD Proteolytic Factor

15 The following is a detailed description of identification and purification of an AD proteolytic factor that includes a Ca^{2+} activated serin protease whose P1 cleaving activity is inhibited by ACT and PN2.

Brain fractions were incubated with the iodinated peptide (^{125}I-P1) and treated with disuccinimidyl suberate ("DSS") to crosslink any proteins

20 that were in intimate contact with the peptide, i.e., to crosslink any enzyme-substrate complex (and, in this instance, any protease-substrate complexes. Then, the enzymes were recovered in fractions containing a labelled enzyme-substrate complex, stabilized by the DSS crosslinking, and N-terminal fragments were detected by autoradiography on TLC plates,

25 generally as described above in the detailed protocol for the cysteine AD protease.

Employing these assays (TLC, DSS crosslinking), a specific serin protease activity was partially purified from Alzheimer's brain homogenates by classical liquid chromatography.

30 Results of an initial purification of specific protease activity from Alzheimer's disease brain ("AD brain") are shown in Fig. 1. Brain

- 12 -

homog nates were prepared in phosphate buffered saline ("PBS") (20 mM phosphate buffer pH 7.0, 0.15 M NaCl) and 1 mM dithi threitol ("DDT"), and spun at 10,000 g. The supernatant was dialyzed against PBS (20 mM phosphate buffer pH 7.0, 20 mM NaCl) and 1 mM DDT, and applied to

5   DE52-cellulose (Whatman) equilibrated with 10 mM Tris-HCl (pH 7.0, 20 mM NaCl) and 1 mM DDT; the column was extensively washed and bound proteins were eluted with 0.5 M NaCl. The active fraction was further purified by ammonium sulfate precipitation, followed by dialysis.

The purification was monitored by a peptide degradation assay as

10   follows. All fractions were incubated with iodinated peptide 1 ("^{125}I-P1", amino acid sequence HSEVKMDAEF) in 10 mM Tris-HCl (pH 7.6, 1 mM CaCl$_2$) for one hour, and then the cleavage products were separated by TLC on cellulose microcrystalline plates (J.T. Baker), followed by autoradiography (panel C). The TLC solvent was n-butanol:pyridine:acetic

15   acid:water (15:10:3:12 by volume) as described generally in P. Tempst *et al.* (1983), *Eur. Jour. Biochem.*, Vol. 135, pp. 321-330. Fractions were also reacted with ^{125}I-P1 for 30 minutes at 4 °C, cross-linked with 0.5 mM DSS for 15 minutes at room temperature, and subjected to SDS-PAGE, and th gel was stained with coomassie blue (panel A), dried and exposed to X-ray

20   film (panel B).

Lane identifications in Fig. 1 are as follows: lane 1, Mr standards; lanes 2, 6, 15, DE52 column flowthrough fraction; lanes 3, 7, 10, ammonium sulfate ("AS") precipitation, 0-25% saturation; lanes 4, 8, 11, 25-50% AS; lanes 5, 9, 12, 50-75% AS; lane 13, .75% AS; lane 14, untreated ^{125}I-P1.

25   The asterisk (*) indicates the minor 30 Kd band. Further purification, including a 100,000 g spin in PBS followed by solubilization of the pell t in 1% Triton X-100 in PBS and a second spin at 100,000 g, revealed that following these treatments the enzymatic activity is found in both the soluble fraction and the membrane-bound fraction.

30   Results of purification of a specific serine protease from AD brain by size exclusion chromatography are shown in Fig. 2. An ammonium sulfate

fraction 50-75%, obtained as described above, was dialyzed, applied to a mono Q column (Pharmacia) and luted with a 20-500 mM NaCl gradi nt in 10 mM Tris HCl, pH 7.0, with 1 mM DDT. Fractions demonstrating activity were concentrated and applied to a Sephadex S-300 column

5   (Pharmacia) and eluted with 10 mM Tris-HCl, pH 7.0, 200 mM NaCl, and 1 mM DDT.

Panels A, B and C in Fig. 2 are as in Fig. 1. Analysis of S-300 column fractions 14 to 32 is shown; fraction numbers are indicated. The first lane in Panel C is untreated ^{125}I-P1; the second lane is fraction AS 50-

10   75% (before S-300 separation). Fractions 23-28 were reserved for further analysis.

A single protein was radioaffinity labeled (Fig. 1) following initial purification. Subsequent steps of purification resulted in a major protein of approximately 68 Kd (Figs. 1 and 2) and a minor one at 30 Kd (Fig. 1).

15   Fig. 3 illustrates effects of inhibitors on the protease activity of the serine AD proteolytic factor isolated as described above. A peptide degradation assay, using 0.1 µg ^{125}I-P1 for each trial as described above with reference to Fig. 1, was used. Lane identifications in Fig. 3 are as follows: lanes 1 and 11, no protease; lanes 10 and 12, the protease fraction

20   alone ("PF"); lanes 2-9 and 13-16, the protease fraction was treated for 15 minutes at room temperature with various agents, then the P1 was added and the mixture incubated at 37 °C for 1 hour (except lane 9, which was incubated for 20 hours); lane 2, PF with PN1, 1 µM; lane 3, PF with ACT, 0.4 µM; lane 4, PF with ACT, 1 µM; lane 5, PF with ACT, 1.5 µM; lane 6,

25   PF with PN2, 0.2 µM; lane 7, PF with PN2, 0.4 µM; lane 8, PF with PN2, 0.75 µM, for 1 hour; lane 9, PF with PN2, 0.75 µM, for 20 hours; lane 13, PF with β-mercaptoethanol, 0.5 mM; lane 14, PF with 2 mM Ca^{2+} ; lan 15, PF with 2 mM EGTA; lane 16, PF with 1 mM DFP.

EGTA, an inhibitor of Ca^{2+} dependent proteases, and DFP, an

30   inhibitor of serin proteases, prevent cleavage of the ^{125}I-P1 by th AD proteolytic factor (Fig.3), indicating that the fraction was nriched in a Ca^{2+}

activated, serin protease. Additional Ca²⁺ nhanc s th d gradation
(Fig. 3, lane 14). In addition, two serine protease inhibitory proteins from
human brain that may be involved in regulating the degradation of β-PP,
ACT (Calbiochem) and purified PN2 (secreted β-PP), also inhibited cleavage

5   of P1 by the AD proteolytic factor. The complex of the AD proteolytic factor
protease with PN2 is reversible; compare lane 8 (1 hr incubation) with lane
9 (20 hrs incubation). In contrast, protease nexin 1 and albumin did n t
influence the activity of the AD proteolytic factor.

The Kunitz-type protease inhibitors are identical to the inhibitor

10   protease nexin 2 ("PN2", described, for example, in W.E. Van Nostrand *et al.*
(1989), *Nature*, Vol. 341, pp. 546-49; T. Oltersdorf *et al.* (1989), *Nature*, Vol.
341, pp. 144-47.

Sequences of the peptide 1 cleavage products generated by
cathepsin G and some other common proteases were compared to thos

15   generated by the serine AD protease fraction; the results of a comparison of
P1 cleavage products for Ca²⁺ activated specific serine protease ("CASP")
from Alzheimer's disease brain and cathepsin G on peptide 1 are shown in
Fig. 4. Five micrograms of unlabeled peptide was incubated with the
enzymes in 10 mM Tris-HCl, pH 7.6, 1 mM CaCl₂ for 15 hours at 37 °C.

20   Resulting mixtures were analyzed by direct peptide sequencing as described
generally in P. Tempst *et al.* (1989), *Anal. Biochem.*, Vol. 183, pp. 290-300;
the percentage cleavage of each peptide bond is indicated under the arrows.
Unseparated proteolytic fragments were directly sequenced as a mixture.
Of the enzymes tested, only cathepsin G and the AD proteolytic fraction

25   cleaved P1 before and after the methionine, and thus only they among these
enzymes are capable of generating the cleavage to release the N-terminus of
the β-protein from the β-protein precursor. Although cleavage kinetics of
the AD proteolytic factor are relatively slow, a presence of active AD
proteolytic factor in brain would ensure accumulation of the β-protein over

30   time. The partially purified AD protease cleaves th Asp-Ala bond (Fig. 4)
which would result in a β-protein missing the N-terminal M thionin .

Cathepsin G is not detectabl in human brain tissue using immunohistoch mistry or Western or northern blot analysis.

The calcium-activated AD proteolytic factor cleaves metabolically labelled endogenous β-PP substrate as well as the P1 peptide. Labelled ^{35}S-

5   β-PP, made as described in S. Sisodia et al. (1990), Science, Vol. 248, pp. 492-95 and obtained from S. Sisodia, was incubated with the AD proteolytic factor, and the fragments were separated on gel and the gel was autoradiographed. A mixture of proteases appear to be present, and on or more than one of these may play a role in an alternative pathway in the

10   brains of persons having abnormal amyloid deposits.

In addition to being able to degrade the synthetic P1, the serine protease fraction also degraded purified human PN2, and metabolically labeled ^{35}Met-PN2 secreted into the medium by cells transfected with th human β-PP770. Other labeled secreted proteins were not affected by th

15   protease.

Thus far, two protease inhibitors have been described that may be involved in amyloid deposits of the β-protein type: β-PP (PN2) (the β-protein precursor) and ACT. In the brain, the two forms of the β-PP, on having the protease inhibitory domain (751/770 amino acids) and the other

20   lacking it (695 amino acids), are found in comparable amounts, in contrast to the β-PP in other organs where the inhibitor form prevails. The different ratios of the two forms may explain the almost unique accumulation of th β-protein type amyloid in the brain, although β-protein antibodies can also label skin, intestine and adrenal sections. When the amounts of the β-PP

25   are compared between AD and controls they do not seem significantly different, but high levels of abnormal degradative forms of β-PP are found in AD neurons and neurites and on Western blots using β-PP antibodies.
Use

In AD, and most likely in normal aging, the β-protein is probably

30   formed as a result f an abnormal proteolytic d gradation of a n rmal protein. The finding that th AD cystein protease cleaves after lysin

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suggests that it may also be able to cleave at amino acids 15-17 (QKL; Q, glutamine; L, lysin) of th β-protein, the normal physiologic site of β-PP processing. Minor changes in the β-PP, an imbalance in proteases and inhibitors may influence many normal brain processes, for instance neurite

5   extension.

    An aberrant proteolytic degradation of the β-PP can contribute to amyloid deposition, which in turn may be trophic or toxic to neurons and astrocytes, causing the neuritic response, neuronal cell death and cognitive deficits. AD may be treated according to the invention by administering to

10   the patient an inhibitor of the AD proteolytic activity according to the invention. Such an inhibitor can be, for example, a competitive inhibitor, such as a fragment of the β-PP molecule corresponding to the binding site of the proteolytic enzyme.

    Likely candidates for effective inhibitors of AD proteolytic activity can

15   be screened by incubating an AD protease according to the invention with a known specific substrate (such as a synthetic oligopeptide having an amino acid sequence corresponding to a sequence spanning the β-protein N-terminus) in the presence of a candidate agent under conditions in which the AD protease would be expected, in the absence of an inhibitor, to cl ave

20   the synthetic oligopeptide near the N-terminus. Candidate agents that effectively inhibit proteolysis in such a trial can then be tested for inhibitory effect in an *in vitro* model and/or in an animal model.

    A preferred inhibitor is capable of crossing the blood-brain barrier, so that it can be administered parenterally or orally. Also, a preferred

25   inhibitor is a molecule other than a peptide, so that the inhibitor will not be rapidly degraded following administration. Also, the preferred inhibitor specifically inhibits the β-PP cleaving activity of the AD protease, and does not generally inhibit the activity of brain proteases that are essential to normal metabolism.

30   Proteolytic fragments resulting from the action on β-PP of an AD protease according to th invention can be detected using, *e.g.*, a battery of

antibodies directed against th C-terminus and the N-terminus of the β-PP on Western blots. Such analysis can aid in establishing where the β-PP molecule is cleaved and how the β-protein may be generated. Once th production of the β-protein is carried out *in vitro*, inhibitors (or agents that

5 are candidates for inhibitors) may be screened for capacity to arrest formation of the AD protease *in vitro*.

Monoclonal antibodies ("mAb") raised against a purified AD prot ase according to the invention, prepared using standard protocols, can be used to establish (and to quantify) the cellular location of the enzyme in the

10 brain and in other tissues such as skin, kidneys, and liver. Such mAb can also be used in the screening of expression libraries, for locating and identifying AD protease-encoding genes, and for cloning such AD protease genes for use in AD protease-producing expression systems.

The damage to tissues resulting from amyloid deposition in AD

15 appears to be irreversible. The neurological symptoms characteristic of AD appear to result from such tissue damage. For a therapy for AD according to the invention to be effective, it should be employed before amyloid deposition has substantially progressed and before neurological symptoms are manifested.

20 Determining the presence of, or monitoring the quantity of, AD proteases can provide a tool for early diagnosis of incipient AD. Tissu r body fluid samples such as, for example, samples of blood, CSF, saliva, urine, can be drawn and assayed for the presence of AD proteases, as an indication of a likelihood of abnormal β-PP metabolism, producing β protein

25 and, ultimately, causing amyloid deposition in tissues.

Other embodiments are within the following claims. For exampl , any crosslinking agent other than DSS can be used in the enzyme purification protocol, provided that the agent is capable of forming crosslinks in an enzyme-substrate complex between portions of the enzyme

30 and the substrate where the nzyme and substrate are in near proximity.

Claims

1. A proteolytic factor capable of cleaving β-protein precursor at a site outside the β-protein domain and near the β-protein N-terminus.

2. The proteolytic factor of claim 1, said proteolytic factor being capable of cleaving β-PP at a site at the β-protein N-terminus.

3. The proteolytic factor of claim 2, said proteolytic factor being capable of cleaving β-PP at a site following a lysine residue or a methionine residue near the β-protein N-terminus.

4. The proteolytic factor of claim 1, said proteolytic factor comprising a serine protease.

5. The proteolytic factor of claim 1, said proteolytic factor comprising a cysteine protease.

6. A method for treating Alzheimer's disease in a patient, comprising reducing β-protein precursor proteolysis at a site near the β-protein N-terminus.

7. The method of claim 5, comprising administering to the patient an inhibitor that inhibits proteolysis outside the β-protein domain of β-PP and at a site near the β-protein N-terminus.

8. The method of claim 6, comprising administering to the patient an inhibitor that inhibits proteolysis in the vicinity of the β-protein N-terminus.

9. The method of claim 5 wherein said inhibitor inhibits proteolytic activity of a proteolytic factor that acts outside the β-protein domain of β-PP at a site near the β-protein N-terminus.

10. The method of claim 5 wherein said inhibitor inhibits proteolytic activity of a proteolytic factor that acts at a site in the vicinity of the β-protein N-terminus.

11. The method of claim 6 wherein said inhibitor is capable of passing the blood-brain barrier *in vivo*.

12. Th method of claim 6 wh rein said inhibitor is administered parenterally or orally.

13. A method for purifying an enzyme from a sample, comprising steps of incubating the sample with a labelled substrate of the enzyme or
5 with a labelled fragment of a substrate to which the enzyme binds, treating the sample with a crosslinking agent to crosslink any enzyme-substrate complexes in the sample, and recovering labelled complexes.

14. A method for diagnosis in a subject of a disease characterized by accumulation of amyloid, comprising determining the level, in a sampl of
10 tissue or body fluid from the subject, of an AD proteolytic factor.

15. A method for screening for an agent useful in treatment of a disease characterized by accumulation of amyloid, comprising incubating an AD protease with a peptide having an amino acid sequence corresponding to the sequence spanning the β-protein N-terminus in the presence of a
15 candidate agent, and determining the degradation of the peptide.

FIG. 1

FIG. 2A

FIG. 2B

FIG. 2C

SUBSTITUTE SHEET

FIG. 3

Cathepsin G　　H　S　E　V　K　M　D　A　E　F
　　　　　　　　　　　　　▲　　　▲　▲　▲
　　　　　　　　　　　　　3　　100　46　7

CASP　　　　　　　H　S　E　V　K　M　D　A　E　F
　　　　　　　　　　▲　　　　▲　▲　▲
　　　　　　　　　　9　　　　21　18　34

FIG. 4

*HSEVKMDAEF
*HSEVK
*HSEVKM

CH PI

O   0.05   0.16   M   NaCl

FIG. 5

←*HSEVKMDAEF

←*HSEVKM

A B C D E

FIG. 6

FIG. 7

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 9/50
US CL: 435/219

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
US CL:	435/219

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

CAS
BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X, P	BIOCHEM. BIOPHYS. RES. COMM., Volume 174, No. 2, issued 31 January 1991, Abraham et al., "A Calcium-Activated Protease from Alheimer's Disease Brain Cleaves at the N-Terminus of the Amyloid β-Protein", pages 790-796, see entire document.	1-4
A, P	BIOCHEM. BIOPHYS. RES. COMM., Volume 175, No. 2, issued 15 March 1991, Matsumoto et al., "Abnormal and Deficient Processing of β-Amyloid Precursor Protein in Familiar Alzheimer's Disease Lymphoblastoid Cells", pages 361-365, see summary and introduction.	1-4
A, P	Chemical Abstracts, Volume 114, No. 9, issued 04 March 1991, Siman et al., "Proteolytic Processing of β-Amyloid Precursor by Calpain I", see page 528, column 1, abstract no. 79578, J. Neurosci., 10(7), 2400-11.	1-4

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 December 1991	20 DEC 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Jon P. Weber

FURTHER INFORMATION CONTINUED FROM THE SEC ND SHEET	
X — Y	J. BIOL. CHEM.. Volume 265. No. 7, issued 05 March 1990, Nelson et al.. "Clipsin, a Chymotrypsin-Like Protease in Rat Brain Which is Irreversibly Inhibited by α-1-Antichymotrypsin", pages 3836-3843. see entire document.	1-4 ___ 1-4
X — Y	PROC. NATL. ACAD. SCI, USA, Volume 87, issued May 1990, Cataldo et al., "Enzymatically Active Lysosomal Proteases are Associated With Amyloid Deposits in Alzheimer Brain", pages 3861-3865, see entire document.	1-4 ___ 1-4

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____ . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☒ Claim numbers 7, 9, 10 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13]. specifically:

Method claims cannot depend from a product claim, and clearly define what what is to be searched. Here, the method appears to add no limitations the product.

3. ☐ Claim numbers _____ . because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment to
Telephone practice Telephone Memorandum

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-4

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

(54) Title: AMYLOIDIN PROTEASE AND USES THEREOF

(57) Abstract

A proteolytic enzyme isolated from human tissue which exhibits a proteolytic activity to hydrolyze Met-Asp peptide bond in an amyloid-like substrate is disclosed. This enzyme has been designated "amyloidin" because it proteolytically cleaves a Met-Asp bond similar to the one present in the amyloid procursor protein to release a fragment having the mature Asp terminus of the β-amyloid peptide. Antibodies to the amyloidin protease is also provided. Methods to isolate and purify the amyloidin protease is provided, as well as assays to screen for inhibitors of the amyloidin protease. Also disclosed is the gene encoding the protease, and methods for expression of the protease by recombinant DNA means.

AMYLOIDIN PROTEASE AND USES THEREOF

Field of the Invention

The present invention relates to purification of
mammalian enzymes and more particularly to the purification of
human amyloidin protease, the identification of the gene
encoding the protease, the identification of inhibitors of
this protease, and various uses thereof.

Background of the Invention

Proteases are enzymes possessing the activity of
hydrolyzing peptide bonds in proteins and polypeptides. One
subclass of proteases, the metalloproteases are dependent on
an integral zinc atom catalysis and often require exogenous
calcium for activity. One such enzyme, which has been
referenced in the literature as collagenase-like peptidase (EC
3.4.99.31), Pz-peptidase (Barrett (1990) Biol Chem Hoppe-
Seyler 371(Supp):311-320) or metalloendopeptidase (EC
3.4.24.15) (Orlowski, et al (1989) Biochem J 261:951-958)
cleaves preferentially bonds on the carboxyl side of
hydrophobic amino acid residues and is believed to function in
the metabolism of bioactive peptides.

Similar enzymatic activity towards collagen sequence-
based peptides have been detected in a number of human tissue
extracts by various investigators; however, most of the work
was confined to the measurement of peptidase activity using
collagen sequence-based peptides (Lessley, et al (1985) J
Androl 6(6):372-378; Rajabi, et al (1984) Am J Obstet Gynecol
150(7):821-826 and Ito, et al (1977) Clin Chim Acta 78(2):267-
270). Pierotti, et al (1990) Biochem 29:10323-10329 recently
report the molecular cloning and primary structure of rat

testes metalloendopeptidase. The enzyme is composed of 645
amino acids with a molecular weight of 72,985 daltons. There
does not appear to be any reports that provide identification
of human Pz-peptidase, either by partial purification and
5   characterization, or by using a battery of substrates or
inhibitor profiles.

Recently, investigators have preliminarily identified a
proteolytic activity from human brain of about 68,000 daltons
that is capable of cleaving between the Met and Asp residues
10   of a small synthetic peptide HSWVKMDAEF, which corresponds to
amino acids 592 through 600 in the ß-amyloid precursor protein
("APP") (Abraham, et al (1990) Neurobiol Aging 11A:303) with
an N-terminal His. When this peptide, having an ^{125}I
radioiodinated His residue, is incubated with "brain protease"
15   fractions, fragments are generated and separated by thin layer
chromatography (TLC). The N-terminal fragments were detected
by exposure of the TLC plate to film (Abraham, et al (1991)
Biochem Biophys Res Comm. 174: 790-796). The cleavage pattern
obtained with the brain protease preparation was primarily at
20   three sites, between the Lys-Met, Met-Asp, and Asp-Ala, with
some cleavage obtained at His-Ser. The peptide cleavage was
inhibited by diisopropylfluorophosphate (DFP), α_1-
antichymotrypsin, and protease nexin II, all of which only
inhibit serine proteases. Based on chemical crosslinking
25   studies with the iodinated peptide substrate, two bands, one
at approximately 68,000 daltons and another at approximately
30,000 daltons, are suggested to be candidates for the
protease(s) in the preparation.

In more recent presentations (Abraham, et al (1991) J
30   Cell Biochem Suppl. 15G:115; Abraham, et al (1991) J Neurochem
57 (Suppl.):5109), these investigators claim at least two
different proteases in the preparation, one being the
previously described calcium-dependent serine protease, and
the other a cysteine metalloprotease. To date, no structure
35   or characterization or any of these proteases has been
presented.

APP is a membrane-spanning glycoprotein that is expressed
in many mammalian tissues and cell lines and is encoded by a

gene that, in humans, is found on chromosome 21. The
ß-amyloid core protein, referred to as the ß- or A4 peptide,
is an approximately 39-42 amino acid long peptide fragment of
APP, and is the major component of the myriad amyloid deposits
5   that accumulate extracellularly in the brains of patients with
Alzheimer's disease (AD) or form the cerebrovascular amyloid
in associated blood vessels.

There are at least three forms of the precursor protein:
APP695 (Kang, et al (1987) Nature 325:733); APP751 (Ponte, et
10   al (1988) Nature 331:525); and APP770 (Kitaguchi, et al (1988)
Nature 331:530) which refer to the number of amino acids in
the primary protein transcript. All of these forms contain
the ß-peptide sequence, which starts 28 amino acids N-terminal
to the beginning of the putative transmembrane region, and
15   ends approximately 14 amino acids in the transmembrane region.
The numbering of amino acids as used herein corresponds to
that used for APP695.

Recent work on the metabolism of the APP in cell culture
has clearly shown that after intracellular maturation of the
20   full transmembrane form of the protein, there is a specific
proteolytic processing event which leads to extracellular
secretion of a large N-terminal region, and leaves behind in
cell membranes a small, C-terminal fragment reactive with
antisera to the carboxyl end of the APP (Oltersdorf, et al
25   (1990) J Biol Chem 265:4492). The size of this C-terminally
reactive fragment made it likely that it contains the entire
ß-peptide. However, characterization by direct protein
sequencing of the N-terminal of this fragment showed that it
starts at Leu17 of the ß-amyloid core peptide where Asp597 of
30   APP695 is counted as Asp1 of the ß-peptide (Esch, et al (1990)
Science 248:1122). Characterization of the soluble secreted
form by isolation of the peptide containing its C-terminal
region also clearly showed that it ends at Gln15. Thus, in
this normal processing pathway, the transmembrane form of APP
35   is cleaved inside the ß-peptide (either before or after Lys16,
which is missing; presumably, it is taken off either by a
carboxypeptidase or an aminopeptidase activity post-cleavage),
and thus this pathway precludes the formation or deposition of

the ß-peptide. It also follows then that an alternative
proteolytic pathway must exist for generation of the
ß-peptide.

The most likely characteristic of such a pathway would be
5   a proteolytic cleavage between Met596 and Asp597, since
protein sequencing of either senile (core) or vascular amyloid
always starts at this aspartic acid residue, although there
has been reported to be a ragged N-terminus for core amyloid
(Masters, et al (1985) Proc Natl Acad Sci USA 82:4245). The
10   preliminary work reported by Abraham et al (1990) supra,
provides some insight as to how APP might be proteolytically
processed to release the ß-amyloid core protein.

Identification of mammalian proteases that are capable of
cleavage at this site is essential in order to screen for
15   inhibitors of such cleavage. Such inhibitors would be useful
for therapeutic intervention in AD.

Cell culture models of the blood brain barrier may be
used for the design of drug delivery systems for the
inhibitors of the present invention. Such cell culture models
20   are disclosed in PCT/US90/05106, filed 13 September 1990 and
PCT/US90/05105, filed 13 September 1990.

Disclosure of the Invention
The present invention provides human amyloidin protease,
capable of cleaving the Met-Asp peptide bond in the peptide
25   N-acetyl-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg (Seq ID
No:1), substantially free of natural contaminants. The
protease has an apparent molecular weight in the range of
about 80,000 daltons as determined by SDS-polyacrylamide gel
electrophoresis (under both reducing and nonreducing
30   conditions).

Methods for the purification of amyloidin protease from
human cells, including blood and brain tissue, are also
provided. This protease may be used as a reagent in methods
for the identification of inhibitors against this protease.
35   Such methods include combining the protease with a putative
inhibitor in the presence of an amyloid-like substrate under
conditions sufficient to cleave the Met-Asp bond; and

-5-

monitoring the reaction to see whether cleavage of the
substrate has occurred.

As an additional aspect of the invention, the amyloidin
protease can be used to raise antibodies, using either another
5   species of animal, such as a rabbit, or a hybridoma cell line.
The resulting antibodies are specific for the amyloidin
protease and can be used in diagnostic tests such as, for
example, an immunoassay, or in immunopurification methods.

Further, it has been found that antigenic potential
10 resides in fragments of the whole amyloidin molecule. Thus,
it is possible to raise antibodies that specifically recognize
an immunogenic epitope of an amyloidin protease using a
fragment of the polypeptide. The resulting antibodies can
themselves be used for immunopurification of the respective
15 protease or in diagnostic assays.

Also provided as an aspect of the invention is the gene
encoding the human amyloidin protease, vectors containing the
gene and host cells transformed with the gene which are
capable of expressing human amyloidin protease.

20 Brief Description of the Drawings

FIG. 1 is a chromatogram of amyloidin taken after the
phenyl-TSK chromatography step. The characteristic three-site
cleavage of N-acetyl-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg
(Seq ID No:1) by the eluted enzyme is shown.
25 FIG. 2 is a Coomassie-stained gel of purified amyloidin.

FIG. 3A and 3B are Western blot analyses of rabbit
polyclonal antisera against an amyloidin protease synthetic
peptide.

FIG. 4 is a Western blot analysis of a number of
30 monoclonal antibodies against the amyloidin protease. RAB =
rabbit polyclonal antisera.

Modes of Carrying Out the Invention
A.   Definitions

"Amyloidin protease" as used herein refers to a native,
35 human proteolytic enzyme which shares some homology to Pz-
peptidase from other mammalian sources. The term also

includes synthetic human amyloidin proteases, i.e., proteins
produced by recombinant DNA means, direct chemical synthesis
or a combination of both. Amyloidin protease is a
polypeptide found, _inter alia_, in brain tissue and in blood.

5   The "amyloidin protease activity" of a protein refers to
a peptide hydrolysis activity selective for a Met-Asp peptide
bond similar to that found at the junction separating the
ß-amyloid core peptide from the amino-terminal region of APP.
This activity can be assayed _in vitro_ by incubating the
10 amyloid protease with a synthetic substrate corresponding
to the peptide sequence including the Met-Asp junction and
determining the extent of cleavage. The amyloid protease
activity predominantly cleaves the Met-Asp bond, although
additional cleavage of certain amino-terminal ß-amyloid core
15 peptide residues is observed with at least one of the amyloid
proteases of the invention. This multiple cleavage activity
may contribute to the formation of the ragged amino-terminus
of the ß-amyloid core peptide originally observed by Masters,
et al (1985), supra.

20   As used herein, "amyloid-like substrate" refers to an
"amyloidogenic" polypeptide derived from the APP which has
substantial homology to the region of the APP spanning the
peptide sequence at the Met-Asp bond located at the
amino-terminus of the ß-amyloid core peptide. The source of
25 the polypeptide includes, but is not limited to, microbially
expressed APP or fragments thereof containing the Met-Asp
cleavage site, endogenous APP present in biological materials
such as cells or mammalian tissue homogenates, and
synthetically produced peptides.

30   A peptide "derived from" a designated polypeptide
sequence refers to a sequence which is comprised of a sequence
of at least 6 amino acids, and preferably at least about 10-12
amino acids corresponding to a region of the designated
polypeptide sequence. "Corresponding" means identical to or
35 exhibiting a minimum of about 60% or more amino acid identity
with the designated sequence. The derived sequence is not
necessarily physically derived from the polypeptide sequence
but may be generated in any manner, including chemical

synthesis or DNA replication of the gene encoding the
polypeptide and microbial expression thereof.

B.   General Method

        The practice of the present invention will employ, unless
5   otherwise indicated, conventional techniques of protein
purification, molecular biology, microbiology and recombinant
DNA techniques, which are within the skill of the art. Such
techniques are explained fully in the literature. See, e.g.,
"Guide to Protein Purification" in Methods in Enzymology (M.P.
10 Deutscher, ed., (1990) Academic Press, Inc.); Sambrook,
Fritsch & Maniatis, Molecular Cloning; A Laboratory Manual,
Second Edition (1989); Oligonucleotide Synthesis (M.J. Gait,
ed., 1984); Nucleic Acid Hybridization (B.D. Hames & S.J.
Higgins, eds., 1984); A Practical Guide to Molecular Cloning
15 (B. Perbal, 1984); PCR Protocols, A Guide to Methods and
Applications (M.A. Innis, et al, eds., (1990) Academic Press,
Inc.); Current Protocols in Molecular Biology (F.M. Ausubel,
et al, eds., (1989) John Wiley & Sons); and additional
publications in the series, Methods in Enzymology (Academic
20 Press, Inc.). All patents, patent applications, and
publications mentioned herein, both supra and infra, are
hereby incorporated by reference.
        The present invention provides substantially purified
human amyloidin protease free from natural contaminants.
25 Purified amyloidin protease allows for the amino acid sequence
to be determined, nucleic acid probes designed and amyloidin
protease genes to be cloned. Once cloned, the amyloidin
protease gene can be used to produce recombinant amyloidin
protease.
30   The identity and characterization of human amyloidin
protease further permits the development of in vitro screening
models for agents which inhibit the cleavage of the Met-Asp
bond in APP. If such cleavage inhibition is successful, β-
amyloid core protein formation is prevented. Thus, this model
35 provides a new and valuable medium with which to explore the
molecular pathogenesis of amyloidosis relevant to AD and to
evaluate potentially therapeutic agents.

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As part of the initial work to identify proteases having
proteolytic specificity for the Met-Asp peptide bond of APP,
the following synthetic decapeptide was designed:
N-acetyl-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg. The

5   C-terminal end can be either a free carboxylate (COOH) or an
amide (CO-NH$_2$).

This peptide spans the putative Met-Asp cleavage site and
is referred to herein as APP592-601. In addition to this
substrate, other amyloid-like substrates may be employed in an

10   inhibition assay to screen for protease activities isolated
from mammalian sources which are capable of cleaving the
Met-Asp peptide bond in this sequence. As taught in the
examples, amyloid-like substrates also may be used to
characterize the substrate specificity of the amyloid

15   proteases described in the present invention. While
endopeptidic cleavage predominates at the Met-Asp bond,
cleavage may also occur at the Asp-Ala and Ala-Glu bonds of
the β-peptide. Endopeptidic cleavage of the amyloid-like
substrate is detected by reverse phase high performance liquid

20   chromatography (HPLC), and the site of cleavage determined by
amino acid analysis of the peptide fragments.

To isolate the amyloid protease activities of the present
invention, extracts of mammalian tissues were made in a
variety of aqueous buffers, such as, for example, Tris,

25   phosphate and HEPES, of about 20-50 mM pH 7.5, at 4-8°C,
using conventional homogenation procedures, such as, for
example, Waring blender or Teflon homogenizer, followed by
repeated centrifugation at 10,000-15,000 x g. The supernatant
soluble fraction from this stage ("low ionic strength") are

30   decanted and the pellet is further extracted with high ionic
strength salt, for example 1 M NaCl, and a detergent, such as
1% Triton X-100. If, however, the amyloidin proteases are to
be isolated from mammalian cells such as erythrocytes,
conventional cell disruption techniques may be employed. Such

35   techniques include homogenization, sonication, osmotic lysis
and pressure cycling. Prior to disruption, the cells may be
first concentrated by filtration, centrifugation, or other
conventional methods.

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The various extracts are generally incubated with the
amyloid-like substrates under a variety of conditions. For an
initial screen, about 25 µl of the extract are incubated with
10 µl of a 2 mg/ml solution of the amyloid-like substrate,
5   along with 10 µl of 1 M Tris-HCl, pH 7.5, and 5 µl of a
solution of water, or 10 to 100 mM $CaCl_2$ or 10 to 100 mM EDTA.
After a 30-60 min incubation of the reaction mixture in a
water bath at 37°C, the reactions are quenched by addition of
multiple volumes of ice-cold ethanol, placed in ice for about
10   20 min and the samples are centrifuged at 15,000 x g for 10
min in an Eppendorf microfuge. The supernatant is removed,
dried under vacuum evaporation and reconstituted with water.
        Aliquots of the sample are analyzed by injection onto a
C18 reversed-phase HPLC column and elution with a 0-60%
15   gradient of 0.1% TFA/acetonitrile, to assess the degradation
of the substrate. Multiple fragments were produced when the
peptide was incubated in the presence of from 0.1 to 10 mM
Ca^{++} ions at a pH range over 6.5 to 8, with soluble fractions
obtained from low-ionic strength extracts. Little peptide
20   cleavage activity was detected under other extraction
conditions, for example, with high salt or detergents.
Analysis of the peptide fragments produced by quantitative
amino acid analysis indicated that the major sites of cleavage
were between the Met-Asp, the Asp-Ala, and the Ala-Glu bonds.
25   The Met-Asp cleaving activity from the crude extract was
isolated for further purification.
        The purification methods referred to herein include a
variety of procedures. Among several types which may be
useful are size fractionation using molecular sieve
30   chromatography; ion exchange chromatography under suitable
conditions; adsorption chromatography using nonspecific
supports, such as hydroxyapatite, silica, alumina, and so
forth; dye-ligand interaction chromatography, such as Cibacron
Blue F3GA-Sepharose, Chromatofocusing®; and also gel-supported
35   electrophoresis. In the case of the amyloidin protease,
hydrophobic interaction chromatography, such as using
phenyl-Sepharose, phenyl-Superose or phenyl-TSK, has been
shown to be particularly useful to separate the amyloid

protease activity from natural contaminants including an
activity which, while not consistently reproducible, shares
the Met-Asp cleavage activity. Hydrophobic interaction
chromatography also serves to provide substantial

5   purification. This procedure separates proteins based on the
hydrophobic properties of the protein, unlike ion exchange
chromatography which separates based on charge properties of
the protein.

        In addition, initial purification of the proteases using

10   ion exchange chromatography (such as with using weak anion
exchangers, for example, DEAE-Sepharose) has been shown to be
a particularly effective procedure to increase the purity of
the amyloidin protease. While the ion exchange chromatography
process is described herein primarily with respect to a

15   cross-linked cellulose having functional diethylaminoethyl
moieties and sold, for example, under the trademark DE52
(Whatman), other resins, particularly other mildly anionic
resins, are suitable for partially purifying amyloidin
protease-containing extracts by ion exchange chromatography.

20   Other suitable resins include but are not limited to
cross-linked dextran having DEAE moieties, and polystyrene
cross-linked with benzene having polyaminoethylene moieties.
When the amyloidin protease is being prepared bacterially or
in some other culture, as will be possible using recombinant

25   DNA procedures, pre-purification steps may be omitted.

        Each of these purification techniques are, in a general
sense, well known in the art, and a detailed description of
the peculiarities of their specific application to the
amyloidin protease is described in the examples below.

30   During the isolation steps, purification of the amyloidin
protease is monitored by testing chromatography fractions for
its ability to cleave the Met-Asp peptide bond in an
amyloid-like substrate as analyzed by RP-HPLC.

        Amyloidin has multiple cleavage sites, although the

35   predominant cleavage site occurs at the Met-Asp peptide bond.
Replacement of amino acid residues at the amino-t rminal
region of the amyloid-like substrate can eliminate or reduce
cleavage at the additional sites as shown in Example 5 herein.

Once substantially purified, the native amyloidin
protease may also be subjected to amino acid sequence
analysis. Applying conventional peptide sequencing
procedures, using for example, an Applied Biosystems model
5   470A gas-phase sequencer amino acid sequences for each of the
amyloidin proteases may be generated.

The amino acid composition of amyloidin is as follows:

Residue	Predicted Composition*
Asx	58.6
Glx	94.2
Ser	31.7
Gly	51.7
His	23.6
Arg	61.0
Thr	32.8
Ala	57.1
Pro	29.1
Tyr	26.0
Val	43.1
Met	16.3
Cys	8.6
Ile	18.7
Leu	83.3
Phe	30.0
Trp	ND
Lys	44.1

*Predicted Composition equals the number of
approximate amino acids; Asx and Glx refer to
(Asp and Asn) and (Glu and Gln), respectively;
Cys was not quantitatively determined; Trp
was not determined.

The purified amyloidin protease can be used to raise
either polyclonal or monoclonal antibodies. The amyloidin
protease is injected into a mammal, such as rabbits, mice or
35   guinea pigs, and the resulting antibodies recovered from the
serum. Alternatively, monoclonal antibodies may be produced
by immunization of mice with either the purified protein or
fragments thereof, and fusion of their splenic cells with
murine myeloma or plasmacytoma cells. These protocols are
40   conventional in the art.

One of the internal peptides of amyloidin has been shown
to be immunogenic. This sequence, as well as other
immunogenic regions, may be produced synthetically using

available amino acid synthesizers. Such immunogenic peptides
contain epitopes, that is, a determinant responsible for
specific interaction with an antibody molecule.

   Antibodies to either the whole amyloidin protease or to
5 immunogenic fragments derived therefrom can be used in
standardized immunoassays, such as radioimmunoassays (RIA) and
enzyme-linked immunosorbent assays (ELISA). In addition, such
antiboies may be used to localize the protease in
immunochemical or immunohistochemical methods, as further
10 described in the examples.

   Once the amino acid sequence is determined, recombinant
DNA encoding the amyloidin protease may be prepared. First,
oligonucleotide probes encoding a portion of the determined
amino acid sequence are prepared and used to screen DNA
15 libraries for the gene encoding the amyloidin protease. The
basic strategies for preparing oligonucleotide probes and DNA
libraries, as well as their screening by nucleic acid
hybridization, are well known to those of ordinary skill in
the art. See, for example, DNA Cloning: Volume I (D.M.
20 Glover, ed. 1985); Nucleic Acid Hybridization, supra; Current
Protocols in Molecular Biology, supra; and Molecular Cloning:
A Laboratory Manual, supra.

   First, a DNA library is prepared. The library can
consist of a genomic DNA library from a selected mammal, such
25 as human. DNA libraries can also be constructed of cDNA
prepared from a poly-A RNA (mRNA) fraction by reverse
transcription. The mRNA is isolated from a cell line or
tissue known to express the amyloidin protease. cDNA (or
genomic DNA) is cloned into a vector suitable for construction
30 of a library. A preferred vector is a bacteriophage vector,
such as phage lambda. The construction of an appropriate
library is within the skill of the art. Alternatively, the
cDNA or genomic library may also be purchased from commercial
sources, for example, Clontech and Stratagene, Inc.

35   Once the library is obtained, oligonucleotides to probe
the library are prepared and used to isolate the desired
amyloidin gene. The particular nucleotide sequences selected
are chosen so as to correspond to the codons encoding a known

amino acid sequence from the amyloidin protease. Since the
genetic code is redundant, it will often be necessary to
synthesize several oligonucleotides to cover all, or a
reasonable number, of the possible nucleotide sequences which
5   encode a particular region of the protein. One can also
design a single probe or "guessmer" wherein one uses codon
bias and other considerations, such as CG dinucleotide
underrepresentations to guess the best sequence, or by using
inosine bases where ambiguity in the sequence exists
10   (Sambrook, et al, supra). It may also be desirable to use two
probes (or sets of probes), each to different regions of the
gene, in a single hybridization experiment. Automated
oligonucleotide synthesis has made the preparation of probes
relatively straightforward. In addition, probes may be
15   commercially obtained.

     Alternatively, one may use the polymerase chain reaction
(PCR) to amplify a portion of the desired gene encoding the
amyloidin protease. In its simplest form, PCR is an _in vitro_
method for the enzymatic synthesis and amplification of
20   specific DNA sequences, using two oligonucleotide primers that
hybridize to opposite strands and flank the region of interest
in the target DNA. A repetitive series of cycles involving
template denaturation, primer annealing, and the extension of
the annealed primers by DNA polymerase results in the
25   exponential accumulation of a specific fragment whose termini
are defined by the 5' ends of the primers. PCR reportedly is
capable of producing a selective enrichment of a specific DNA
sequence by a factor of 10°. The PCR method is described in
Saiki, et al (1985) _Science 230_:1350 and is the subject of
30   U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159. The
portion of the amyloidin gene synthesized by the PCR technique
will be used to probe cDNA libraries for clones encoding the
full length amyloidin cDNA.

     Because the genetic code is redundant, PCR from known
35   amino acid sequence requires PCR with either degenerate,
inosine substituted, or "guessmer" PCR oligos. (See PCR
Protocols, Innis, et al, supra, especially the chapter on
"Degenerate Primers for DNA Amplification" at pp. 39-45;

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Sambrook, et al, supra; Maisonpiere, et al (1990), _Science_
_247_:1446; Hohn, et al (1990) _Nature 344_:339. These
techniques have been widely used to clone a variety of genes
as described in the above references. One can use PCR to
5   amplify DNA sequences from either cDNA generated from RNA,
genomic DNA or from a cDNA or genomic library. Strategies
using either conventional PCR as described above, or "anchor"
PCR could be used. In anchor PCR, one uses a library
containing the amyloidin gene as the PCR substrate, and uses
10   one sequence within the amyloidin gene and another within the
vector that the library is in so that the region amplified
contains sequences from the vector as well as from the
amyloidin gene. In this case only very limited amino acid
information is necessary. PCR conditions and components such
15   as temperatures, concentrations of magnesium, _Taq_ polymerase,
and oligos would be optimized as described in Innis, et al,
supra. One might also utilize conditions where 7-deazaguanine
is used to allow the amplification of sequences containing
secondary structure.
20   As an alternative to cloning the gene based on nucleic
acid probes, one can use the amino acid sequence of amyloidin
to prepare antibody probes that can be used to screen for the
amyloidin gene. Given the amino acid sequence, peptides of
identical sequence can by synthesized by standard techniques,
25   and these peptides can be used to immunize rabbits or mice.
Polyclonal or monoclonal antibodies to either amyloidin or
peptides derived therefrom can be generated and used to detect
amyloidin clones from an appropriate library. Libraries made
in vectors which are designed to express the gene of interest,
30   include but not limited to lambda gt11, lambdaZAP, or
lambdaORF8 (see Ausubel, et al, supra, and Sambrook, et al,
supra) can be screened with an antibody to that library.
Libraries in these vectors can be generated or purchased from
sources such as Clontech or Stratagene. The protein of the
35   cloned gene is expressed in these vectors and the ability of
an antibody to bind to the expressed protein allows one to
identify the amyloidin clone by standard antibody probing
techniques.

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A DNA molecule containing the coding sequence for
amyloidin protease can be cloned in any suitable vector and
thereby maintained in a composition substantially free of
vectors that contain the coding sequence of other mammalian
5   genes, including those encoding other amyloidin protease
activities. Numerous cloning vectors are known to those of
skill in the art, and the selection of an appropriate cloning
vector is a matter of choice.

For expression of the amyloidin protease, a variety of
10   systems can be used, including, but not limited to, bacterial,
yeast, insect, and mammalian systems.

Bacterial expression vectors such as pEx12Mcr and
pEx10mer (Seedorf, et al (1987) EMBO J 6:139) and a variety of
vectors discussed in the above cited publications, can be used
15   to express fusion proteins that contain amyloidin sequences
linked to bacterial genes. Other bacterial expression vectors
can be used to make intact full-length amyloidin in bacterial
cells. For bacterial expression the vector needs to have a
bacterial promoter and a ribosome binding site.

20   Mammalian vectors useful in the present invention
include, but are not limited to pORFex13 (Bernard, et al
(1987) EMBO J 6:133), pL1, pcDV1, pcD-X (all from Okayama and
Berg (1983) Mol Cell Biol 3:280), pSV2 and derivatives thereof
including pSVneo and pSVdhfr (Sambrook, et al, supra), pRSVneo
25   (Gorman, et al (1983) Science 221:551 and vectors derived from
these and/or related vectors such as pRSVcat (Gorman, et al
(1982) Proc Natl Acad Sci USA 79:6777) can be used to express
amyloidin in a variety of mammalian cell types. The amyloidin
gene is placed in these vectors in operable juxtaposition and
30   then put into animal cells by standard techniques. Once
inside the cell the protein is expressed from these vectors
containing the amyloidin gene. Such expression control
elements for expression in animal cells include a promoter,
enhancer, splice site (this is optional) and polyadenylation
35   sequences. A variety of systems are available for xpression
in animal virus systems, such as, for example, bovine papiloma
virus, retroviruses, SV40 and other viruses as described in
Ausubel, et al, supra and Sambrook, et al, supra, and in "High

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Level Production of Proteins in Mammalian Cells" by Randal J. Kaufman, in Genetic Engineering (1987) vol 9:155-198, Jane K. Stelow, ed.

5   The insect virus system based on baculovirus vectors can also be used to express the amyloidin gene. Insect virus systems are commercially available from Invitrogen Corp., San Diego, CA. Such vectors include, but are not limited to pAc373 ("A Manual for Methods for Baculovirus Vectors and Insect Cell Culture Procedures" by Max Summers and Gale Smith,

10   published by Texas Agricultural Experiment Station), pVL941 (Luckow and Summers, (1989) Virology 170:31). These vectors can be used to transfer the amyloidin gene into a baculovirus and the recombinant virus thus obtained used to infect insect cells. The infected insect cells are used to produce

15   amyloidin protein. Complete methods for these procedures are described in Summers, et al, supra.

The amyloidin protease of the present invention can be used to develop and/or identify agents which inhibit the cleavage of the Met-Asp bond similar to that found in APP.

20   The selection of appropriate inhibitor molecules will generally be guided by the rate at which the test compound inhibits cleavage of the amyloid-like substrate. Inhibition assays may be developed to assess the inhibitory spectrum of various test compounds on the cleavage of the amyloid-like

25   substrate in the presence of the amyloidin protease.

A suitably modified amyloid-like substrate may be incubated, under conditions of neutral pH in a suitable aqueous buffer, with a protease that has been incubated with a potential inhibitory compound (at room temperature for 15-30

30   min, for example), for 1-4 hr, or a period of time sufficient to obtain significant endopeptidic cleavage of the substrate in the absence of the inhibitory agent. The proportion of cleavage is then quantitated. Suitably varying the concentration of the inhibitor compound and measuring the

35   inhibition of cleavage as compared to zero inhibitor concentration, will allow one to determine an inhibition curve, from which the inhibitory efficacy, such as the inhibitor concentration at which 50% of the enzyme's cleavage

activity is inhibited, or the inhibition constant (Ki) can be calculated by standard methods.

The present invention also provides the discovery that "clipsin", a chymotrypsin-like protease, selectively cleaves the amyloid substrate APP592-601 at the Met-Asp peptide bond. Clipsin was first reported by Nelson and Siman (1990) <u>J Biol Chem 265</u>:3836, and was partially purified from rat brain. These investigators showed that clipsin was relatively specific for the APP, but they did not identify any specific cleavage site. Also reported was the specificity of additional known proteins, such as calpain, for APP.

As shown herein, selectivity for the Met-Asp peptide bond persists even when clipsin is subjected to a further purification step (chromatography on a soybean trypsin inhibitor affinity column). These data are not obvious in view of the strong Suc-Ala-Ala-Pro-Phe-pNa (K_{cat}/K_u = 57,000) and weak Suc-Ala-Ala-Pro-Met-pNA (K_{cat}/K_u = 5,200) cleavage activity of clipsin previously reported. While the Nelson and Siman paper dismissed the possibility that clipsin might be one of the identified rat mast cell proteases, RMCP I and II (Woodbury, et al (1981) <u>Methods in Enzymol 80</u>:588), comparison of the enzymatic properties of clipsin with authentic RMCP I and with human skin chymase (the analogous human mast cell enzyme disclosed in Schechter, et al (1986) <u>J Immunol 137</u>:962) using the amyloid-like substrate APP592-601, clearly showed that all three proteases cleaved the APP592-601 peptide at the Met-Asp bond. Nelson, et al (1990) <u>Soc Neuroscience Abstr 16</u>:788) have recently reported more complete purification of "clipsin", including the partial amino acid sequence from the N-terminus, which indicate that clipsin indeed is RMCP I.

RMCP I, RMCP II and human skin chymase are known to belong to a family of related chymotrypsin-like proteases, also called "chymases." Members of this family include, for example, mouse mast cell proteases 1-6 (Reynolds, et al (1990) <u>Proc Natl Acad Sci USA 87</u>:3230-3234) and possibly, human cathepsin G. Each of th se mammalian proteases which exhibit the Met-Asp cleavage activity may be considered equivalents

for purposes of testing inhibitory agents of the amyloidin
protease of the present invention.

    For analysis of cleavage inhibition, the amyloid-like
substrate may be labeled, as described below, by incorporating

5 moieties detectable by spectroscopic, photochemical,
biochemical, immunochemical, or chemical means. The method of
linking or conjugating the label to the amyloid-like substrate
depends, of course, on the type of label(s) used and the
position of the label on the substrate.

10   A variety of labels which would be appropriate for use in
the invention, as well as methods for their inclusion in the
substrate, are known in the art and include, but are not
limited to, enzymes (e.g., alkaline phosphatase and
horseradish peroxidase) and enzyme substrates, radioactive

15 atoms, fluorescent dyes, chromophores, chemiluminescent
labels, ligands having specific binding partners, or any other
labels that may interact with each other to enhance, alter, or
diminish a signal.

    "Specific binding partner" refers to a protein capable of

20 binding a ligand molecule with high specificity, as for
example in the case of an antigen and a monoclonal antibody
specific therefor. These types of binding partners are also
referred to in the art as "capture" labels. Other specific
binding partners include biotin and avidin or streptavidin,

25 IgG and protein A, and the numerous receptor-ligand couples
known in the art. It should be understood that the above
description is not meant to categorize the various labels into
distinct classes, as the same label may serve in several
different modes. For example, ^{125}I may serve as a radioactive

30 label or as an electron-dense reagent. HRP may serve as
enzyme or as antigen for a monoclonal antibody.

    Further, one may combine various labels for a desired
effect. In some situations it may be desirable to use two
labels on a single substrate with due consideration given for

35 maintaining an appropriate spacing of the labels to permit the
separation of the labels during hydrolysis of the peptide
bond. For example, one might label a substrate at its
N-terminus with biotin and its C-terminus with a radioactive

label.   One would detect cleavage of the substrate by passing
the reaction mixture over or through a solid phase extractant
(SPE) containing avidin or streptavidin.   The SPE is monitored
to assess whether the signal of the C-terminal label changes.
5   Any decrease in signal intensity is an indication of cleavage
inhibition.   Other permutations and possibilities will be
readily apparent to those of ordinary skill in the art, and
are considered as equivalents within the scope of the instant
invention.

10   C.   Examples

The examples presented below are intended to be
illustrative of the various methods and compositions of the
invention.

EXAMPLES

15   Example 1:   Purification Scheme
A.   Protein Purification from Brain Tissue
Frozen human brain tissue (500 g wet weight) was thawed,
then homogenized in a Waring blender with three parts (v/w) of
ice-cold 20 mM Tris, pH 7.5, 2 mM EDTA, 5 mM 2-mercaptoethanol
20   ("Buffer A").   The mixture was centrifuged at 10,000 x g for
60 min at 4°C, and the pellet discarded   The supernatant was
recentrifuged at 15,000 x g for 60 min.
About 1 l of the centrifuged solution was applied to a
100 ml packed DE-52 diethylaminoethyl anion exchange column
25   pre-equilibrated with Buffer A.   After loading for 8 hr at
4°C, the column was washed with 10 volumes of 1 l of Buffer A,
then eluted with 60 mM NaCl in Buffer A.   Fractions of the
eluate containing peptide cleavage activities were pooled and
concentrated in an Amicon pressure cell to 5 ml.
30   The concentrated eluate was chromatographed on a 2.5 x
100 cm S-200 Sephacryl (Pharmacia) molecular sieving column,
equilibrated with Buffer A supplemented with 100 mM NaCl, and
chromatographed overnight at 4°C at 1 ml/min.   All three
peptide cleavage activities eluted in a symmetrical peak with
35   an apparent MW of 80,000.   The fractions containing peptide

cleaving activity were pooled, then dialyzed for 16 hr at 4°C
into 10 mM sodium phosphate, pH 7.5, containing 10 uM Ca⁺⁺.

The dialyzate was then loaded on to a Bio-Gel HT column
(Bio-Rad) (40 ml bed volume) pre-equilibrated with the same

5   buffer used for dialysis at 1 ml/min at 4°C, washed with five
volumes of the buffer, and then eluted with a 250 ml 10 to 250
mM sodium phosphate linear gradient. The protease activity
eluted at approximately 100 mM sodium phosphate. The sharp
peak of activity was pooled, made 1 M in ammonium sulfate (by

10   dilution with 3 M ammonium sulfate in 100 mM sodium phosphate,
pH 7.5) centrifuged at 15,000 x g at 4°C for 20 min, and the
supernatant loaded at 4°C onto a 7.5 x 75 mm HPLC Phenyl-TSK
column equilibrated in 1 M ammonium sulfate in 100 mM sodium
phosphate, pH 7.5. The column was washed with 10 ml of the

15   equilibration buffer, and eluted using a linear gradient in
which the ammonium sulfate concentration decreased from 1 M to
0 M. The amyloidin fractions eluted at approximately 0.4 M
ammonium sulfate and were pooled.

The purification steps described here are important,

20   since at least one major contaminating protease activity is
separated out at the Phenyl-TSK step. This activity, which is
not affected by Ca⁺⁺, and cleaves the peptide at the Ala-Glu
bond, elutes early from the column, followed by the
characteristic three-site cleavage pattern of amyloidin. The

25   chromatogram shown in FIG. 1 and developed from the material
eluted from the Phenyl-TSK HPLC column, shows the
characteristic three-site cleavage by amyloidin. Although we
were able to detect an activity which we designated "amyloidin
II" that also cleaved the substrate at the Met-Asp bond, this

30   activity could not be reproducibly isolated.

The pooled material was dialyzed at 4°C for 4 hr against
25 mM bis-Tris, pH 6.3 and then applied to a 1.5 x 30 cm PBE
94 Chromatofocusing® column (Pharmacia) pre-equilibrated with
the 25 mM bis-Tris, pH 6.3 buffer. The column was eluted with

35   a decreasing pH gradient from 6.3 to 3.8 using Polybuffer 74
diluted 1/8 with water, and adjusted to pH 3.8 with HCl.
Amyloidin fractions eluted at an approximate pH of 4.3.
SDS-PAGE (reducing conditions) analysis of fractions

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containing amyloidin activity showed a predominant band at
80,000, as estimated by the relative mobility against low
molecular weight protein markers purchased from Bio-Rad
(Richmond, CA).

5   B.   Protein Purification of Amyloidin from Blood
         Outdated blood was obtained from the blood bank.   Six
units of whole blood were centrifuged at 2,000 x g for 30 min,
and the plasma and buffy coat discarded.   The packed cells
were washed four times with Buffer A plus 140 mM NaCl, with
10   centrifugation at 2,000 x g for 30 min and discarding the wash
after each step.   The collected, washed erythrocytes were
lysed by osmotic shock in 6 volumes of 5 mM Tris-HCl, pH 7.5,
2 mM EDTA and 5 mM 2-mercaptoethanol, for 30 min on ice, then
centrifuged at 15,000 x g for 60 min at 4°C.
15   The supernatant was mixed with DE-52 (Whatman)
pre-equilibrated with Buffer A using approximately 85 ml of
settled bed volume for the ion exchanger per unit of whole
blood.   The DE-52 slurry was stirred for 1 h on ice, then
washed in a Buchner funnel with 8 L of the Buffer A solution.
20   The washed ion exchanger was packed into a glass column,
washed with 4 L of Buffer A, then eluted with 60 mM NaCl in
Buffer A.
         The pool of peptide-cleavage activity was dialyzed
against 4 L of 10 mM sodium phosphate, pH 7.5, 10 μM CaCl$_2$,
25   and the dialyzate passed through an Affigel Blue column (50 ml
bed volume, Bio-Rad).   All the peptide cleavage activity
passed through the column unretarded.   The solution was
collected and loaded onto a 100 ml Bio-Gel HT column
pre-equilibrated with the dialyzing buffer.   The column was
30   then washed with 100 ml of the same buffer and eluted with a
linear gradient of 10-250 mM sodium phosphate, pH 7.5, 10 μM
CaCl$_2$, total volume 600 ml.   The peak of peptide cleavage
activity was pooled, then dialyzed against 25 mM Bis-Tris, pH
6.3, and applied to a 1.5 x 40 cm PBE 94 (Pharmacia)
35   Chromatofocusing column.
         The Chromatofocusing column was washed with 200 ml of
the loading buffer and the retentant eluted with a decreasing

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pH gradient from 6.3 to 3.8, using Polybuffer 74 diluted 1:8
with water and then the pH was adjusted to 3.8 with HCl. The
amyloidin activity (eluting at approximately pH 4.3) was
pooled, and then dialyzed against 100 mM sodium phosphate, pH

5   7.5, 1 M ammonium sulfate. The dialyzate was then loaded onto
a 7.5 mm x 75 mm HPLC Phenyl-TSK column (Toyo-Soda, Japan)
equilibrated with the dialysis buffer, washed with 10 ml of
the equilibration buffer, then eluted using a linear gradient
in which the ammonium sulfate concentration decreased from 1 M

10   to 0 M. The amyloidin fractions eluted at approximately 0.4 M
ammonium sulfate and were pooled.

        C. Alternative Protein Purification Scheme
        Following initial tissue extration procedures as
described for brain tissue (1A) or blocd (1B) above, the

15   soluble extract was mixed with DE-52 (Whatman) pre-
equilibrated with 20 mM Tris, pH 7.5, 2 mM EDTA, 5 mM
ß-mercaptoethanol, using approximately 100 ml packed resin per
liter of extract.

        Peptide cleavage activity was pooled and dialyzed against

20   4 1 of 10 mM sodium pohosphate, pH 7.5, 10 μM CaCl$_2$, then
applied to a 100 ml Bio-Gel HT column pre-equilibrated with
the dialyzing buffer. The elution of this column and
subsequent steps, e.g., PBE 94 (Pharmacia) Chromatofocusing®
and HPLC phenyl-TSK chromatography, were done exactly as

25   described in Example 1B above. The exact order of these
subsequent steps may be performed in a different sequence than
that presentedin this example. When phenyl-TSK chromatography
preceded PBE 94 Chromatofocusing®, similar purification and
yields were obtained.

Fraction	Volume (ml)	[Protein] (mg/ml)	Total Protein (mg)	Total Activity DAEFR area, mV-sec/min	% Yield	Specific Activity (area/min/mg protein)	Fold Purification
Crude	4200	236	991200	114371	100.00%	0.12	1
DE52	165	10.1	1666.5	422334	369.27%	253.43	2196
HT	142	1.4	198.8	69262	61.81%	298.05	2583
PBE 94	150	0.54	81	62125	54.32%	766.98	6647
Phe TSK	40	0.14	5.6	11644	10.18%	2079.29	18020

30   D. Enzymatic Assay
        The peptides of the invention can be prepared by solid
phase synthesis (Kent and Lewis in "Synthetic Peptides in

Biology and Medicine," Alitalo, ed. (1985) Elsevier) or by
other standard peptide synthetic means. APP592-601 was
synthesized by Applied Biosystems (Foster City, CA) and the
anhydrous hydrogen fluoride (HF)-crude further purified by

5   reverse-phase HPLC. The composition was confirmed by amino
acid analysis on an Applied Biosystems 420 Automated Amino
Acid Analyzer. APP592-601 has a free carboxy terminus which
is preferred for purposes of the cleavage assay described
below, whereas the analogs synthesized below have a C-terminal

10   amide.

Routinely, 25 μl of enzyme solution were mixed with 10 μl
of 1 M Tris, pH 7.5, 5 μl 10 mM CaCl$_2$, and 10 μl of a 2 mg/ml
stock solution of APP592-601 in water, or 20 mM Tris, pH 7.5,
0.15 M NaCl, in 1.5 ml polypropylene microfuge tubes. The

15   reaction mixtures were incubated for 60 min at 37°C in a water
bath, then quenched with 450 μl ice-cold ethanol, and
incubated on ice for a further 20 min. They were centrifuged
at 15,000 x g for 10 min, and the supernatants transferred to
new polypropylene tubes and dried under vacuum. The residue

20   was dissolved in 0.5 ml water, centrifuged at 15,000 x g for 5
min, and 200 μl of the supernatant injected onto a 0.46 x 30
cm Vydac C18 column equilibrated with 0.1% trifluoroacetic
acid in water, at 1 ml/min. The column was immediately eluted
with a linear gradient to 40% acetronitrile in 0.1%

25   trifluoroacetic acid in water, over 20 minutes. The elution
was monitored at 220 nm, and peaks were individually
collected, hydrolyzed in 6 N HCl at 65°C for 2 hr, then
subjected to quantitative amino-acid analysis in an Applied
Biosystems amino-acid analyzer. Once identified, times of

30   elution were used to identify cleavage patterns. The
characteristic three-site cleavage pattern of amyloidin is
shown in FIG. 1.

Example 2:   Enzymatic Properties
Amyloidin is strongly inhibited by EDTA, since

35   substitution of 5 μl 100 mM EDTA for the CaCl$_2$ in the standard
peptide cleavage assay described in Example 1D, leads to no
detectable cleavage of the APP592-601 by this protein.

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The molecular weight estimate for the purified amyloidin was made by comparing the mobility of the Coomassie-stained band with that of low molecular weight standard protein markers (FIG. 2, Lanes 1 and 4) supplied by Bio-Rad
5   (phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400); Lane 2 is amyloidin (non-reducing); and Lane 3 is amyloidin (reducing).

The inhibition of amyloidin by various inhibitors was
10   tested by individually pre-incubating the enzyme with the inhibitor compounds listed in the following table for 30 min at room temperature, prior to addition of APP592-601 to start the reaction. The conditions for each reaction are also provided in the table. The solvent stock solution of
15   inhibitor is made up in water unless otherwise indicated (DMSO, dimethylsufoxide; EtOH, ethanol). Percent of activity of amyloidin is shown, as calculated with respect to control without inhibitor but with appropriate solvent.

                    Inhibition of Amyloidin

Inhibitor*	Stock conc.	Assay conc.	% Control Activity
PMSF	50 mM EtOH	1 mM	69
DFP	20 mM EtOH	2.5 mM	0
EDTA	100 mM	10 mM	0
E-64	10 μg/ml	1 μg/ml	119
1,10-phen.	5 mM DMSO	0.1 mM	75
phosphoramidon	1 mg/ml	0.1 mg/ml	79
Calp. Inh. II	2.5 mg/ml	50 μg/ml	130
chymostatin	1 mg/ml DMSO	25 μg/ml	77
aprotinin	0.1 mg/ml	2 μg/ml	102
α1-PI	1 mg/ml	30 μg/ml	98
α1-ACT	1 mg/ml	30 μg/ml	121

* PMSF, phenylmethylsulfonyl fluoride; DFP, Diisopropyl-fluorophosphate; EDTA, ethylenediaminetetraacetic acid; 1,10-
35   phen., 1,10-phenanthroline; calp. inh. II, calpain inhibitor II; α1-PI, α1-proteinase inhibitor; α1-ACT, α1-antichymotrypsin.

Calpain inhibitor-II or E-64 (both strong inhibitors of the Ca^{++}-dependent cysteine protease, calpain), had no
40   inhibitory effect on amyloidin. 1,10-phenanthroline and phosphoramidon, both strong inhibitors of metalloproteases, were only weakly inhibitory (25% inhibition). Neither

α-1-proteinase inhibitor or α-1-antichymotrypsin, two general
plasma serine proteinase inhibitors, had any inhibitory effect
on amyloidin, nor did aprotinin, the bovine Kunitz trypsin
inhibitor.

5   Example 3:   Structural Characterization
          A.   Amyloidin
          To obtain sequence information, approximately 300 pico-
     moles of amyloidin purified from human brain tissue (Example
     1) were electrophoresed on a 7.5% acrylamide gel using
10   SDS-PAGE, and the protein band was visualized with Poinceau
     Red (stock solution from Sigma diluted 1:10 with water).
          The band was excised from the gel and minced into small
     pieces with a clean razor blade.   30 picomoles of Lys-C
     endopeptidase (Boehringer Mannheim) in 100 μ 50 mM Tris-HCl,
15   pH 8.5, 1 mM EDTA, was then added to the protein, and
     incubated overnight.   The gel pieces were repeatedly extracted
     with 10 mM ammonium bicarbonate in acetonitrile (5 x 100 μ),
     the washings combined and dried.   The dried gel samples were
     then taken up in 50 μ 0.1% TFA, and injected into a 0.2 cm x
20   15 cm Vydac C18 micropore column, and eluted with a linear
     gradient of 0-60% acetonitrile over 60 min.   The three peaks
     that were deemed most pure were then sequenced to completion
     or to whatever was practical on an Applied Biosystems 470A
     automated protein sequencer, with online PTH analysis, using
25   the programs supplied by the manufacturer.
          The three most prominent peaks were sequenced and are
     provided below:
          H₂N-Arg-Val-Tyr-Asp-Gln-Val-Gly-Thr-Gln-Glu-Phe-
     Glu-Asp-Val-Ser-Tyr-Glu-Ser-Thr-Leu-Lys-COOH (Seq. ID No.2);
30   H₂N-Val-Asp-Gln-Ala-Leu-His-Thr-Gln-Thr-Asp-Ala-
     Asp-Pro-Ala-Glu-Glu-Tyr-Ala-Arg-Leu-Cys-Gln-Glu-Ile-Leu-Gly-
     Val-Pro-Ala-Thr (Seq. ID No:3); and
          H₂N-Glu-Tyr-Phe-Pro-Val-Gln-Val-Val-Thr-His-Gly-
     Leu-Leu-Gly-Ile-Tyr-Gln-Glu-Leu-Leu-Gly-Leu-Ala-Phe-His-His
35   (Seq. ID No:4).

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Direct attempts at sequencing the 50,000 MW band, by transferring to Immobilon membranes (Millipore) were unsuccessful, which indicated a probable blocked N-terminus.

Example 4:   Substrate Specificity

5   The ability of amyloidin to cleave short peptide-based para-nitroanilide substrates was tested by incubating 10 μ of a 20 mM stock of peptide p-NA substrate with 25 μ of enzyme, 20 μ of 100 mM CaCl$_2$, 40 μ of 1 M Tris-HCl, pH 7.5, and 105 μ water, in 96-well microtiter plates, and monitoring for

10   increase in absorbance at 405 nm, in a Molecular Devices Vmax Kinetic Microplate Reader.   The results are provided below. No measurable increase in A$_{405}$ was detected, even after incubations up to 2 hours.   Thus, amyloidin does not appear to cleave the pNA substrates tested, including one which is

15   derived from the APP592-601 sequence.

Substrate*	Amyloidin
Suc-AAPM-pNA	0
MeS-AAPV-pNA	0
Suc-AAPF-pNA	0
Ac-AD-pNA	0
Boc-AAd-Pna	0
Z-AA-pNA	0
Z-RR-pNA	0
Z-RK-pNA	0
Ac-EVKM-PNA	0

* Sub-AAPM-, Sucinyl-alanyl-prolyl-methionyl-; MeS-AAPV-, Methoxysuccinyl-alanyl-alanyl-prolyl-valyl-; Suc-AAPF-, Succinyl-alanyl-alanyl-prolyl-phenylalanyl-; Ac-AD-, Acetyl-alanyl-aspartyl-; Boc-AAD-, Butyloxycarbonyl-alanyl-
30   alanyl-aspartyl-; Z-AA-, benzyloxycarbonyl-alanyl-alanyl-; Z-RR-, benzyloxycarbonyl- arginyl-arginyl-; Z-RK-, benzyloxycarbonyl-arginyl-lysyl-; Ac-EVKM-, acetyl-glutamyl-valyl-lysyl-methinyl-pNA; pNA-, para-nitroaniline

Amyloidin does not appear to cleave oligopeptide

35   para-nitroanilide substrates, including one based on the APP sequence after which it cleaves in the APP592-601 peptide.
        Various peptides were purchased from Bachem (Torrance, CA) and incubated with the human amyloidin protease using the conditions described in Example 1D.   Cleavage products were

40   isolated by reverse-phase HPLC, and subjected to amino acid analysis to determine site(s) of cleavage.   The following table show the results btained with amyloidin protease compared to reported site(s) of cleavage by Pz-peptidase.

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Substrate	Cleavage Products
Bradykinin	<u>ArgProProGlyPhe</u> <u>SerProPheArg</u>
Neurotensin	<u>GluLeuTyrGluAspLysProArg</u> <u>ArgProTyrIleLeu</u>
LH-RH	<u>GluHis</u> <u>TrpSerTyr</u> <u>GlyLeuArgProGlyNH$_2$</u>
	_____ (minor)
Dynorphin A108	<u>TyrGlyGlyPheLeu</u> <u>ArgArgIle</u>
	_____(minor)

The spaces indicate cleavage sites of Pz-peptidase reported by
Barrett (1990), supra. Underlined peptides are those
identified following cleavage by the amyloidin of the present
invention.

Example 5:   Subsite Requirements
        In order to test the subsite requirements of amyloidin in
the APP592-601 cleavage assay, analogs of this peptide
substrate were synthesized.
    A.   Chemical Synthesis of (N-acetyl)-APP (592-601)
          CONH$_2$ analogs
        The peptide corresponding to residues 592 to 601 of the
695 APP was synthesized on the Applied Biosystems Model 430A
Peptide Synthesizer using the t-boc methodology. All
boc-amino acids and synthesis reagents were purchased from
Applied Biosystems Inc., and the amino acid side chain
protecting groups are as follows: Arg(TOS), Asp(OBzl),
Lys(2ClZ), and Ser(OBzl). Ala, Met, Phe, Gln, Nleu and Val
were used with no side chain protecting group. [(TOS) -
tosyl, (OBzl) - O benzyl, (2ClZ) - 2,6-dichlorocarbobenzoxy].
The software for controlling the synthetic cycle was designed
specifically for making long chain peptides and peptides with
sequence specific coupling difficulties. The general cycle is
as follows: boc deprotection, neutralization; amino acid
activation, 1st coupling (in CH$_2$Cl$_2$), neutralization; amino
acid activation; 2nd coupling (in dimethylformamide),
neutralization; and finally acetic anhydride capping.
        Boc-amino acids were activated as preformed symmetric
anhydrides by addition of 0.5 equivalents of
N,N-dicyclohexylcarbodiimide (DCC) with the exception of
boc-Arg(TOS) which was activated to its corresponding

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HOBT-ester by addition of 1 equivalent of
1-hydroxybenzotriazole and 1 equivalent of DCC. After the
second coupling of each amino acid, any uncoupled amine
remaining was capped by addition of acetic anhydride in CH_2Cl_2

5   plus a catalytic amount of diisopropylethylamine (DIEA). The
capping step is to prevent synthesis of deleted peptide
sequences which are often difficult to separate from the
target peptide during purification.

After addition of the N-terminal amino acid, the boc

10   group was removed using 50% (TFA) in CH_2Cl_2 and neutralized
with a solution of 10% DIEA in CH_2Cl_2. The exposed primary
amine of the N-terminal amino acid was then acetylated using
the same protocol using the capping step.

The solid support, p-methylbenzhydrilamine resin, was

15   purchased from Fisher Biotech. Treatment of the fully
protected peptide resin with anhydrous HF, cleaved the peptide
from the solid support, removed all the side chain protecting
groups, and produced the crude peptide product as the
C-terminal carboxy amide derivative.

20   The crude peptide was purified to >98.0% purity using
preparative scale reverse phase chromatography on a Vydac,
C18, 330A, 10um column with dimensions of 2.2 cm x 25 cm in
length. The crude peptide was dissolved and loaded onto the
column in 5%, [0.1% TFA/CH_3CN]/H_2O and eluted using a linear

25   gradient of 5% to 50% [B] over 135 minutes. ([B] = 0.1%
TFA/CH_3CN).

The structural integrity of the purified peptide was
assessed by analytical HPLC, amino acid composition analysis,
and mass spectrometry.

30   Following synthesis and purification, the peptides were
then incubated with amyloidin under conditions identical to
those developed with APP592-601. The results are summarized
below. The underlined residues designated changes from the
native peptide sequence while the arrows indicate peptide

35   cleavage sites.

    ↓ ↓ ↓
Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg
       ↓
Ser-Glu-Val-<u>Gln</u>-Met-Asp-Ala-Glu-Phe-Arg
      ↓ ↓
Ser-Glu-Val-Lys-<u>Ala</u>-Asp-Ala-Glu-Phe-Arg
     ↓ ↓ ↓
Ser-Glu-Val-<u>Arg</u>-Met-Asp-Ala-Glu-Phe-<u>Lys</u>
     ↓ ↓ ↓ ↓
Ser-Glu-Val-Lys-<u>Nle</u>-Asp-Ala-Glu-Phe-Arg

  With the exception of the analog in which Lys595 was
replaced with a Gln, amyloidin cleaved at multiple sites,
which differed from peptide to peptide. The replacement of
Met596 by Ala resulted in loss of cleavage at this site, but
cleavage shifted to the Lys595-Ala596 bond. In the Gln595
analog, all the cleavage by amyloidin was at the Met-Asp bond.
  While amyloidin has been demonstrated to make the
relevant cleavage at the Met-Asp bond of synthetic amyloidin
substrates, under certain preliminary conditions tested, the
protease did not cleave the full-length protein (APP) produced
by baculovirus cells transformed with the gene encoding APP.
However, this does not preclude the involvement of this enzyme
in the processing of APP as amyloidin may be only one of
several enzymes whose combined activity is necessary for the
generation of the β-peptide from APP.

Example 6: <u>Purification of Clipsin/RMCP I</u>
  The procedure to isolate RMCP I was adapted exactly from
that described by Nelson & Siman (1990), supra.
However, after the final extraction of the membrane pellets
with 50 mM HEPES, pH 7.5, 1 M MgCl₂, and 0.1% Brij 35, the
soluble extract was not further dialyzed, but loaded onto an
immobilized soybean trypsin inhibitor column. This protocol
and the subsequent steps were adapted from that taught from
the purification of RMCP I described in Woodburg, et al,
supra.

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The soybean trypsin inhibitor column was prepared as
follows: 15 mg of soybean trypsin inhibitor (Sigma Chemicals)
was dissolved in 10 ml of 0.1 M sodium bicarbonate, pH 8.5,
and mixed with 3 g of washed activated CH-Sepharose (Sigma)
5   for 2 h end-over-end in a plastic tube. The gel slurry was
then filtered over a coarse sintered glass funnel, and
extensively washed with alternating buffers, 0.1 M formate, pH
3, and 0.2 M Tris-HCl, pH 8. The washed gel was re-suspended
in 0.2 M Tris, pH 8, then equilibrated with 50 volumes of the
10   same buffer after loading in a small glass column. The gel
was then equilibrated with 50 mM HEPES, pH 7.5, 1 M $MgCl_2$, and
0.1% Brij 35. The RMCP I extract was then loaded onto the
column, and the flow-through material re-applied three times.
(Measurement of Suc-Ala-Ala-Pro-Phe-pNA hydrolysis, which was
15   used by Nelson & Siman to assay for "clipsin" activity,
indicated that 95% of this activity had bound to the matrix.)
The column was then washed with 5 volumes of the loading
buffer, then eluted with 10 ml of 25 mM formate, pH 3, 0.1
mg/ml bovine serum albumin. The eluate was collected into an
20   equal volume of 0.2 M ammonium bicarbonate, pH 8.6, with 0.1
mg/ml BSA. This pool was diluted 1:1 with distilled water and
treated with 1 g of reagent grade barium sulfate (Aldrich) for
30 min on ice. The supernatant was decanted and the pellet
washed with 5 ml of 10 mM Tris, pH 8. The pellet was
25   extracted with 2 ml of 20 mM Tris, pH 8 and 1 M NaCl. This
extract was assayed for both Suc-Ala-Ala-Pro-Phe-pNA
hydrolysing activity, as well as APP592-601 cleaving activity.
APP592-601 was cleaved at the Met-Asp bond, and this protease
activity was completely inhibited by pre-treating the protease
30   aliquot with 200 ng of α_1-antichymotrypsin, which had been
shown to be a strong inhibitor of RMCP I. This pre-treatment
also completely eliminated the measured
Suc-Ala-Ala-Pro-Phe-pNA hydrolysing activity.
Incubation of 200 ng each of RMCP I and human skin
35   chymase (HSC), obtained from Dr. Norman Schecter, University
of Pennsylvania, with APP592-601 in the standard peptide
cleavage assay also resulted in cleavage at the Met-Asp bond.
Since the latter two enzymes are known to hydrolyze

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Suc-Ala-Ala-Pro-Phe-pNA, and are also inhibitable by
α$_1$-antichymotrypsin, this suggested that RMCP I and HSC all
belong to a second class of proteases, distinct from
amyloidin, but also able to cleave the APP592-601 at the
5   Met-Asp peptide bond.

Example 7:   Production of Antibodies
        For the generation of antibodies to amyloidin, two
approaches were taken.   A peptide sequence corresponding to
sequence 1 obtained from structural analysis of amyloidin, but
10   having an additional Cys at the amino-terminus was synthesized
by the standard synthesis methods described earlier.   About 72
mg of the crude HF-cleaved peptide
(Cys-Val-Tyr-Asp-Gln-Val-Gly-Thr-Gln-Glu-Phe-Glu-Asp-Val-Ser-
Tyr-Glu-Ser-Thr-Leu-Lys) were conjugated to 12 mg of rabbit
15   serum albumin (RSA), using Sulfo-MBS (Pierce, USA) in 50 mM
potassium phosphate, pH 7 (Lerner, et al (1981) Proc Natl Acad
Sci USA 78:3403-3407).   The conjugation reaction was allowed
to proceed for 20 min on ice, then immediately separated on a
Sephadex G-10 column.   The conjugated protein pool was sent to
20   Josmin Laboratories (Berkeley, CA) for injection into rabbits.
Briefly, primary immunization was done with 0.5 mg of
conjugated RSA, followed by a first boost after three weeks.
The first bleed obtained two weeks after the last of three
weekly injections was tested by Western blot analysis for
25   reaction against purified amyloidin.   Strong reaction was
obtained in the anti-serum from one of the two rabbits
injected, and this serum was further processed by isolating a
crude IgG fraction by sodium sulfate fractionation.   As shown
in FIG. 3A and 3B, a Western blot using the IgG fraction
30   diluted 1:500 as the primary antibody, the 80,000 dalton
protein band in amyloidin is exclusively recognized.   Lane 1
(FIG. 3A) shows pre-stained Bio-Rad low molecular weight
markers and Lane 2 (FIG. 3B) shows Amersham Rainbow markers;
Lane 2 (FIG. 3A) and Lane 1 (FIG. 3B) show the purified
35   amyloidin.
        In the second approach, 60 μg of purified amyloidin were
electrophoresed on SDS-PAGE, and the 80,000 dalton amyloidin

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band visualized by Rapid Reversible Stain (Diversified
Biotech). The protein band was excised with a razor blade,
then destained according to the manufacturer's instructions.
The gel strip was then cut into small pieces and homogenized

5   with a small amount of phosphate buffered saline (PBS) and
emulsified into a water-in-oil emulsion with Freund's complete
adjuvant by repeated passage through an emulsifying needle.
Aliquots (20 μg) of this were injected into three Balb/C mice.
This procedure was repeated every other week, with the

10   modification that subsequent emulsifications were done with
Freund's incomplete adjuvant. The serum of each mouse was
tested for reactivity against purified amyloidin by Western
blot analysis after three injections. The mouse whose serum
showed the strongest reactivity was further selected for

15   fusion with myeloma cells to generate hybridomas producing
antibodies against amyloidin. The hybridomas were generated
by standard murine fusion procedures as described in
ANTIBODIES: A Laboratory Manual by Harlow & Lane (Cold Spring
Harbor Laboratories, 1988). Briefly, the immunized mouse was

20   sacrificed and the spleen removed. Mixed splenocytes were
obtained by pressing the spleen between frosted ends of glass
slides. These were fused with SP2/0Ag14 plasmacytoma cells
(ATCC No. CRL1581) at a fusion ratio of 1:3 in Dulbecco's
modified Eagle's media (DMEM), supplemented with 20% fetal

25   bovine serum (FBS), 2 mM glutamine, 15 mM HEPES and 0.1 mM
hypoxanthine. Hybridomas were selected for by growing the
cells in the presence of azaserine supplemented DMEM,
augmented with hypoxanthine and 20% FBS. Hybridomas were
screened for reactivity against purified human brain amyloidin

30   using an ELISA. Positives were further tested in Western
blots and the results are shown in FIG. 4. From this single
source, 10 monoclonals have been generated which recognize
purified amyloidin strongly on Western blots.

Example 8: Immunohistological Studies

35   Brain tissue from Alzheimer disease (AD) patients and age
matched controls was immersion fixed in 4.0% paraformaldehyde
in 0.1M phosphate buffer and cut into 4C μm sections on a

sliding microtome. Sections were collected in 0.1M phosphate
buffer and quenched for endogenous peroxidase activity for 20
minutes in 0.3% hydrogen peroxide and 0.5% Triton X-100 in
0.1M phosphate buffer. They were blocked for 1.0 hour in 5.0%
5   milk in phosphate buffer and then incubated for 24 hours in
the polyclonal antibody to amyloidin protease diluted 1:20 in
1.5% goat serum and phosphate buffer. As controls, some
sections were incubated at the same concentration with
preimmune sera from the rabbit producing the antibody and
10 antibody adsorbed with the amyloidin peptide. They were then
processed for immunocytochemistry using standard procedures
for the goat anti rabbit IgG Vectastain ABC kit (Vector
Laboratories). Brain tissue from rats transcardially perfused
with 9.9% NaCl followed by PLP fixative was similarly
15 processed. In addition, similar tissue from rat, AD and age
matched control brains was embedded in paraffin and cut into 8
μm sections. These sections were baked for 1-3 hours and
hydrated by passing the sections for 3 minutes each in three
changes of xylene, two changes of 100% ETOH, and one change
20 each through 95%, 70%, 50% EtOH and dH$_2$O). These were also
routinely processed with the Vectastain ABC kit. To determine
the efficacy of formic acid pretreatment, some of the paraffin
embedded material was incubated with formic acid for 3 minutes
following hydration.

25   In both normal and AD brains the antibody stained large
neurons in layers III and IV, white matter astrocytes, and
smooth muscle cells. In AD brains large reactive astrocytes
were stained in the gray matter. This staining was not seen
with preimmune sera and was blocked by adsorbing the antibody
30 with the peptide antigen. In rat brain, a number of
astrocytes in the hippocampus were labeled; no neurons were
labeled.

    In paraffin embedded AD sections without formic acid
pretreatment only smooth muscle cells and a few, presumably
35 reactive, astrocytes in the gray matter wer labeled; no
obvious pathology was stained. However after formic acid
pretreatment, the predominant staining in the same brains was
associated with AD pathology; dystrophic n urites, notably

those forming neuritic plaques were labeled. Several large
neurons were also labeled, however they were not found evenly
distributed throughout the gray matter. Instead they were
found in clusters, usually in layer V. Smooth muscle cells
5   were also labeled.

In rat paraffin embedded material, no cortical neurons
were labeled before or after pretreatment with formic acid.
Only smooth muscle cells were seen in cortical arterioles,
little or no labeling was seen in the cerebellum. A few
10 neurons were labeled in the midbrain.

In summary, the staining patterns of the polyclonal
antibody against amyloidin protease indicate that it may be
abundant in human brain cells and may be highly expressed in
the cellular components underlying the pathology associated
15 with Alzheimer's Disease. The reason why formic acid
pretreatment changes the staining pattern in paraffin embedded
in tissue is unclear; the compromised epitope may be altered
or obscured in dystrophic versus normal cells and neurites.
The lack of neuronal staining in the rat is interesting and
20 may signify a difference in degradation products between the
two species.

Example 9:   <u>Cloning of the Gene Encoding Human Amyloidin</u>
Total RNA was extracted from normal human brain (patient
ID 87-5); superior temporal gyrus and a human embryonic kidney
25 cell line; 293 (ATCC No. CRL1573). As shown in Example 1,
human brain homogenates have amyloidin activity. Similarly,
293 cell extracts were shown to have amyloidin activity and
therefore cDNA made from this RNA would be a preferred
template for amyloidin sequence identification.
30 Complementary DNA was generated using random hexamers.
PCR primers were designed to give the longest PCR product of
region III (Seq. ID No:4). The sequence of the PCR primers
(EJ-87 and EJ-88) were based on Lathe's rules ((1985) <u>J Mol
Biol 183</u>:1-12) although degenerate primers did work also (EJ-
35 91, EJ-92 and EJ-93).

    EJ-87: 5' TCGAATTC AAG GAG TAC TTC CCT GT 3' (Seq ID No:7
    EJ-88: 5' CAAAGCTT TG GAA GGC CAG GCC CAG 3' (Seq ID No:8)

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EJ-89: 5' CAT GGC CTG CTG GGC ATC TAC CAG GAG 3' (Seq ID No:9)

EJ-91: 5' TCGAATTC AAR GAR TAY TTY CCN GT 3' (Seq ID No:10)

EJ-92: 5' CAAAGCTT RTG RTT NGC NAG NCC 3' (Seq ID No:11)

EJ-93: 5' CAAAGCTT RTG RTT NGC YAA NCC 3' (Seq ID No:12)

5   where N can be A, T, C or G; R can be A or G; and Y can be C
or T.

PCR products were analyzed by Southern blot hybridization
using internal probe EJ-89. PCR reactions were performed at
95°C; 1 min denaturing, 42°C, 48°C or 54°C; 1 min annealing;

10   72°C, 1 min extension times for 35 cycles. A 93 bp product of
the EJ-87/88 reaction that did hybridize with EJ-89 was
excised from an analytical acrylamide gel and used as template
for a re-PCR reaction. DNA sequencing was performed using EJ-
87 and EJ-88 as primers as described by Smith, et al (1990)

15   Biotechniques 9:51. A unique DNA sequence of approximately 40
base pairs was then provided for further cloning efforts.

This 40 base pairs of unique sequence, along with
flanking sequence from the PCR oligos EJ-87 (Seq ID No:7) and
EJ-88 (Seq ID No:8), were used to generate an oligo probe,

20   designated 811 (Seq ID No:13) 5' AAGGAGTACT TCCCTGTGCA
GGTGGTCACG CACGGGCTGC TGGGCATCTA CCAGGAGCTC CTGGGCCTGG
CCTTC 3' to screen libraries for amyloidin clones.

Because this probe contains at least 40 contiguous base
pairs of exact sequence, stringent conditions (55°C, 0.1X SSC

25   wash) were used during screening; otherwise screening
techniques were standard (see Sambrook, et al (1990)). This
probe was used to screen a temporal cortex cDNA library
(obtained from Stratagene, catalogue number 935205), and one
clone (clone 19) was obtained. Partial sequence generated

30   from clone 19 was used to design the following PCR oligos:

895 (Seq ID No:14) 5' GAAATGCACG TGCCTGAG 3'

889 (Seq ID No:15) 5' CCAGGACATA GTCGGCG 3' (antisense)

that were used to generate a double-stranded PCR probe from
the 5' end of clone 19. Although the Stratagene library was

35   screened with this probe (Seq ID No:16) 5' GAAATGCACG
TGCCTGAGAC CAGGAGGAAA GTGGAGGAGG CCTTCAACTG CCGGTGCAAG
GAGGAGAACT GCGCTATCCT CAAGGAGCTG GTGACGCTGC GGGCCCAGAA
GTCCCGCCTG CTGGGGTTCC ACACGCACGC CGACTATGTC CTGG 3', no clones

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containing the 5' end of the coding region of amyloidin were obtained.

Western analysis showed that HeLa cells also contain amyloidin. A standard HeLa cell random primed cDNA library in
5   the lambda gt10 vector was provided by Dr. Bernhard Luscher (University of California, Berkeley). Commercially available HeLa cell cDNA libraries are available from Stratagene (catalogue number 936201) and Clontech (catalogue number HL1022b). This library was screened with the same PCR probe
10   generated from oligos 889 and 895 described above, and 30 positive clones were plaque purified. The amounts of 5' and 3' flanking sequences in all of the positive clones were estimated by PCR analysis using oligos homologous to the lambda vector sequences (from Clontech, catalogue number 5411-
15   1) and either an oligo made to the 5' end of clone 19 (antisense strand; oligo 909; 5' ACTTTCCTCCTGGTCTCA 3') (Seq ID No:17) or to the 3' end of clone 19 (coding strand; oligo 905; 5' GGAGAAGCTCATTGAGTC 3') (Seq ID No:18). Sizing of the PCR products was done by agarose electrophoresis, and those
20   clones with the most flanking sequences were chosen for sequence analysis. These clones were cut out of the lambda vector and subcloned into M13 for sequencing. Two clones, clones cHL57 and cHL53, which together span the entire coding region of amyloidin, were chosen for complete sequence
25   analysis.

The coding region of human amyloidin is provided as Seq ID No:6. The complete sequence of the human amyloidin gene was obtained by sequencing two clones: clone cHL57 provided the nucleotide sequence encoding amino acid residues 1 through
30   480, with Met1 of Seq ID No:5 being the putative initiation codon; clone cHL53 provided the nucleotide sequence encoding amino acid residues 56 thorough the stop condon of the amyloidin protease. Examination of the nucleotide sequence reveals that there is no clear hydrophobic leader sequence as
35   would be expected for a secreted or membrane-bound protein. This is similar to that found by Pierotti, et al (1990), supra for the rat Pz-peptidase. However, the human amyloidin sequence, in contrast to the rat Pz-peptidase sequence,

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contains approximately 132 base pairs of additional coding
DNA, and therefore codes for an enzyme having approximately 44
additional amino acid residues at the carboxy-terminus.

　　　　The open reading frame of the cDNA encoding human
5　amyloidin is composed of 2070 nucleotides, including the stop
codon (Seq ID No:6), encoding a protein with 689 amino acids
residues (Seq ID No:5).　Like other members of the family of
zinc-dependent metallopeptidases, human amyloidin contains the
typical amino acid sequence at and around the active site that
10　is represented by the motif Xaa-His-Glu-Phe-Gly-His-Xaa, in
which the two histidine residues coordinate the Zn^{2+} in the
active center and the glutamate is involved in bond-breaking
process.

　　　　　　　　　　　　　SEQUENCE LISTING
15
　　　(1) GENERAL INFORMATION:
　　　　　　　(i) APPLICANT:　Dovey, Harry F.
　　　　　　　　　　　　　　　　Seubert, Peter
　　　　　　　　　　　　　　　　Sinha, Sukanto
20　　　　　　　　　　　　　　　McConlogue, Lisa C.
　　　　　　　　　　　　　　　　Little, Sheila P.
　　　　　　　　　　　　　　　　Johnstone, Edward M.
　　　　　　(ii) TITLE OF INVENTION: Amyloidin Protease and Uses
　　　　　　　　　　　　　　　　　　　　Thereof
25　　　(iii) NUMBER OF SEQUENCES: 12
　　　　　　(iv) CORRESPONDENCE ADDRESS:
　　　　　　　　　(A) ADDRESSEE: Athena Neurosciences, Inc.
　　　　　　　　　(B) STREET: 800F Gateway Blvd.
　　　　　　　　　(C) CITY: South San Francisco
30　　　　　　　　　(D) STATE: California
　　　　　　　　　(E) COUNTRY: USA
　　　　　　　　　(F) ZIP: 94080
　　　　　　　(v) COMPUTER READABLE FORM:
　　　　　　　　　(A) MEDIUM TYPE: Floppy disk
35　　　　　　　　　(B) COMPUTER: IBM PC compatible
　　　　　　　　　(C) OPERATING SYSTEM: PC-DOS/MS-DOS
　　　　　　　　　(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
　　　　　　(vi) CURRENT APPLICATION DATA:
　　　　　　　　　(A) APPLICATION NUMBER: US
40　　　　　　　　　(B) FILING DATE:
　　　　　　　　　(C) CLASSIFICATION:
　　　　(viii) ATTORNEY/AGENT INFORMATION:
　　　　　　　　　(A) NAME: Murphy, Lisabeth Feix
　　　　　　　　　(B) REGISTRATION NUMBER: 31547
45　　　　　　　　　(C) REFERENCE/DOCKET NUMBER: 17796-002
　　　　　　(ix) TELECOMMUNICATION INFORMATION:
　　　　　　　　　(A) TELEPHONE: (415) 877-0900
　　　　　　　　　(B) TELEFAX: (415) 877-8370

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(2) INFORMATION FOR SEQ ID NO:1:
         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 10 amino acids
             (B) TYPE: amino acid
             (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: protein
       (iii) HYPOTHETICAL: NO
        (iv) ANTI-SENSE: NO
         (v) FRAGMENT TYPE: internal
        (ix) FEATURE:
             (A) NAME/KEY: Modified-site
             (B) LOCATION: 4
             (D) OTHER INFORMATION: /label= Xaa4
                    /note= "Xaa4 can be either Gln or Lys"
        (ix) FEATURE:
             (A) NAME/KEY: Modified-site
             (B) LOCATION: 5
             (D) OTHER INFORMATION: /label= Xaa5
                    /note= "Xaa5 can be either Met or Nle"
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

        Ser Glu Val Xaa Xaa Asp Ala Glu Phe Arg
        1   5   10

(2) INFORMATION FOR SEQ ID NO:2:
         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 21 amino acids
             (B) TYPE: amino acid
             (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: protein
       (iii) HYPOTHETICAL: NO
        (iv) ANTI-SENSE: NO
         (v) FRAGMENT TYPE: internal
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

        Arg Val Tyr Asp Gln Val Gly Thr Gln Glu Phe Glu Asp Val Ser Tyr
        1   5   10   15

        Glu Ser Thr Leu Lys
                    20

(2) INFORMATION FOR SEQ ID NO:3:
         (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 30 amino acids
             (B) TYPE: amino acid
             (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: protein
       (iii) HYPOTHETICAL: NO
        (iv) ANTI-SENSE: NO
         (v) FRAGMENT TYPE: internal
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

        Val Asp Gln Ala Leu His Thr Gln Thr Asp Ala Asp Pro Ala Glu Glu
        1   5   10   15

        Tyr Ala Arg Leu Cys Gln Glu Ile Leu Gly Val Pro Ala Thr
                    20   25   30

(2) INFORMATION FOR SEQ ID NO:4:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: protein
   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (v) FRAGMENT TYPE: internal
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Glu Tyr Phe Pro Val Gln Val Val Thr His Gly Leu Leu Gly Ile Tyr
1               5               10                  15
Gln Glu Leu Leu Gly Leu Ala Phe His His
         20                  25
```

(2) INFORMATION FOR SEQ ID NO:5:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 689 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: protein
   (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Lys Pro Pro Ala Ala Cys Ala Gly Asp Met Ala Asp Ala Ala Ser
1               5               10                  15

Pro Cys Ser Val Val Asn Asp Leu Arg Trp Asp Leu Ser Ala Gln Gln
         20                  25                  30

Ile Glu Glu Arg Thr Arg Glu Leu Ile Glu Gln Thr Lys Arg Val Tyr
         35                  40                  45

Asp Gln Val Gly Thr Gln Glu Phe Glu Asp Val Ser Tyr Glu Ser Thr
         50                  55                  60

Leu Lys Ala Leu Ala Asp Val Glu Val Thr Tyr Thr Val Gln Arg Asn
65                  70                  75                  80

Ile Leu Asp Phe Pro Gln His Val Ser Pro Ser Lys Asp Ile Arg Thr
                 85                  90                  95

Ala Ser Thr Glu Ala Asp Lys Lys Leu Ser Glu Phe Asp Val Glu Met
                 100                 105                 110

Ser Met Arg Glu Asp Val Tyr Gln Arg Ile Val Trp Leu Gln Glu Lys
                 115                 120                 125

Val Gln Lys Asp Ser Leu Arg Pro Glu Ala Ala Arg Tyr Leu Glu Arg
                 130                 135                 140

Leu Ile Lys Leu Gly Arg Arg Asn Gly Leu His Leu Pro Arg Glu Thr
                 145                 150                 155                 160
```

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```
            Gln Glu Asn Ile Lys Arg Ile Lys Lys Leu Ser Leu Leu Cys Ile
                        165             170             175

  5         Asp Phe Asn Lys Asn Leu Asn Glu Asp Thr Thr Phe Leu Pro Phe Thr
                        180             185             190

            Leu Gln Glu Leu Gly Gly Leu Pro Glu Asp Phe Leu Asn Ser Leu Glu
                        195             200             205

 10         Lys Met Glu Asp Gly Lys Leu Lys Val Thr Leu Lys Tyr Pro His Tyr
                    210             215             220

            Phe Pro Leu Leu Lys Lys Cys His Val Pro Glu Thr Arg Arg Lys Val
                225             230             235             240

 15         Glu Glu Ala Phe Asn Cys Arg Cys Lys Glu Glu Asn Cys Ala Ile Leu
                        245             250             255

            Lys Glu Leu Val Thr Leu Arg Ala Gln Lys Ser Arg Leu Leu Gly Phe
                        260             265             270

 20         His Thr His Ala Asp Tyr Val Leu Glu Met Asn Met Ala Lys Thr Ser
                    275             280             285

            Gln Thr Val Ala Thr Phe Leu Asp Glu Leu Ala Gln Lys Leu Lys Pro
                    290             295             300

 25         Leu Gly Glu Gln Glu Arg Ala Val Ile Leu Glu Leu Lys Arg Ala Glu
                305             310             315             320

 30         Cys Glu Arg Arg Gly Leu Pro Phe Asp Gly Arg Ile Arg Ala Trp Asp
                            325             330             335

            Met Arg Tyr Tyr Met Asn Gln Val Glu Glu Thr Arg Tyr Cys Val Asp
                        340             345             350

 35         Gln Asn Leu Leu Lys Glu Tyr Phe Pro Val Gln Val Val Thr His Gly
                        355             360             365

 40         Leu Leu Gly Ile Tyr Gln Glu Leu Leu Gly Leu Ala Phe His His Glu
                        370             375             380

            Glu Gly Ala Ser Ala Trp His Glu Asp Val Arg Leu Tyr Thr Ala Arg
                385             390             395             400

 45         Asp Ala Ala Ser Gly Glu Val Val Gly Lys Phe Tyr Leu Asp Leu Tyr
                            405             410             415

            Pro Arg Glu Gly Lys Tyr Gly His Ala Ala Cys Phe Gly Leu Gln Pro
                        420             425             430

 50         Gly Cys Leu Arg Gln Asp Gly Ser Arg Gln Ile Ala Ile Ala Ala Met
                        435             440             445

 55         Val Ala Asn Phe Thr Lys Pro Thr Ala Asp Ala Pro Ser Leu Leu Gln
                    450             455             460
```

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His Asp Glu Val Glu Thr Tyr Phe His Glu Phe Gly His Val Met His
465   470   475   480

Gln Leu Cys Ser Gln Ala Glu Phe Ala Met Phe Ser Gly Thr His Val
            485   490   495

Glu Arg Asp Phe Val Glu Ala Pro Ser Gln Met Leu Glu Asn Trp Val
        500   505   510

Trp Glu Gln Glu Pro Leu Leu Arg Met Ser Arg His Tyr Arg Thr Gly
        515   520   525

Ser Ala Val Pro Arg Glu Leu Leu Glu Lys Leu Ile Glu Ser Arg Gln
        530   535   540

Ala Asn Thr Gly Leu Phe Ser Leu Arg Gln Ile Val Leu Ala Lys Val
545   550   555   560

Asp Gln Ala Leu His Thr Gln Thr Asp Ala Asp Pro Ala Glu Glu Tyr
            565   570   575

Ala Arg Leu Cys Gln Glu Ile Leu Gly Val Pro Ala Thr Pro Gly Thr
            580   585   590

Asn Met Pro Ala Thr Phe Gly His Leu Ala Gly Gly Tyr Asp Ala Gln
            595   600   605

Tyr Tyr Gly Tyr Leu Trp Ser Glu Val Tyr Ser Met Asp Met Phe His
            610   615   620

Thr Arg Phe Lys Gln Glu Gly Val Leu Asn Ser Lys Val Gly Met Asp
625   630   635   640

Tyr Arg Ser Cys Ile Leu Arg Pro Gly Gly Ser Glu Asp Ala Ser Ala
            645   650   655

Met Leu Arg Arg Phe Leu Gly Arg Asp Pro Lys Gln Asp Ala Phe Leu
            660   665   670

Leu Ser Lys Gly Leu Gln Val Gly Gly Cys Glu Pro Glu Pro Gln Val
            675   680   685

Cys

(2) INFORMATION FOR SEQ ID NO:6:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 2070 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
ATGAAGCCCC CCGCAGCCTG TGCAGGAGAC ATGGCGGACG CAGCATCTCC GTGCTCTGTG     60

GTAAACGACC TGCGGTGGGA CCTGAGTGCC CAGCAGATAG AGGAGCGCAC CAGGGAGCTC    120

ATCGAGCAGA CCAAGCGCGT GTATGACCAG GTTGGCACCC AGGAGTTTGA GGACGTGTCC    180

TACGAGAGCA CGCTCAAGGC GCTGGCCGAT GTGGAGGTCA CCTACACAGT TCAGAGGAAT    240

ATCCTTGACT TCCCCCAGCA TGTTTCCCCC TCCAAGGACA TCCGGACAGC CAGCACAGAG    300

GCCGACAAGA AGCTCTCTGA GTTCGACGTG GAGATGAGCA TGACGGAGGA CGTGTACCAG    360

AGGATCGTGT GGCTCCAGGA GAAAGTTCAG AAGGACTCAC TGAGGCCCGA GGCTGCGCGG    420

TACCTGGAGC GGCTAATCAA GCTGGGCCGG AGAAATGGGC TTCACCTCCC CAGAGAGACT    480

CAGGAAAACA TCAAACGCAT CAAGAAGAAG CTGAGCCTTC TGTGCATCGA CTTCAACAAG    540

AACCTGAACG AGGACACGAC CTTCCTGCCC TTCACGCTCC AGGAGCTAGG AGGGCTCCCC    600

GAGGACTTTC TGAACTCCCT GGAGAAGATG GAGGACGGCA AGTTGAAGGT CACCCTCAAG    660

TACCCCCATT ACTTCCCCCT CCTGAAGAAA TGCCACGTGC CTGAGACCAG GAGGAAAGTG    720

GAGGAGGCCT TCAACTGCCG GTGCAAGGAG GAGAACTGCG CTATCCTCAA GGAGCTGGTG    780

ACGCTGCGGG CCCAGAAGTC CCGCCTGCTG GGGTTCCACA CGCACGCCGA CTATGTCCTG    840

GAGATGAACA TGGCCAAGAC CAGCCAGACC GTGGCCACCT TCCTAGATGA GCTGGCGCAG    900

AAGCTGAAGC CCCTGGGGGA GCAGGAGCGT GCGGTGATTC TGGAGCTGAA GCGTGCGGAG    960

TGCGAGCGCC GGGGCCTGCC CTTCGACGGC CGCATCCGTG CCTGGGACAT GCGCTACTAC   1020

ATGAACCAGG TGGAGGAGAC GCGCTACTGC GTGGACCAGA ACCTGCTCAA GGAGTACTTC   1080

CCCGTGCAGG TGGTCACGCA CGGGCTGCTG GGCATCTACC AGGAGCTCCT GGGGCTGGCC   1140

TTCCACCACG AGGAGGGCGC CAGTGCCTGG CATGAGGACG TGCGGCTCTA CACCGCGAGG   1200

GACGCGGCCT CGGGGGAGGT GGTCGGCAAG TTCTACCTGG ACCTGTACCC GCGGGAAGGA   1260

AAGTACGGGC ACGCGGCCTG CTTTGGCCTG CAGCCCGGCT GCCTGCGGCA GGATGGGAGC   1320

CGCCAGATCG CCATCGCGGC CATGGTGGCC AACTTCACCA AGCCCACAGC CGACGCGCCC   1380

TCGCTGCTGC AGCATGACGA GGTGGAGACC TACTTCCATG AGTTCGGCCA CGTGATGCAC   1440

CAGCTCTGCT CCCAGGCGGA GTTCGCCATG TTCAGCGGGA CCCACGTGGA GCGGGACTTT   1500

GTGGAGGCGC CGTCGCAGAT GCTGGAGAAC TGGGTGTGGG AGCAGGAGCC GCTGCTGCGG   1560

ATGTCGCGGC ACTACCGCAC AGGCAGCGCC GTGCCCCGGG AGCTCCTGGA GAAGCTCATT   1620

GAGTCCCGGC AGGCCAACAC AGGCCTCTTC AGCCTGCGCC AGATCGTCCT CGCCAAGGTG   1680

GACCAGGCCC TGCACACGCA GACGGACGCA GACCCCGCCG AGGAGTATGC GCGGCTCTGC   1740
```

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CAGGAGATCC TCGGGGTCCC GGCCACGCCA GGAACCAACA TGCCTGCAAC CTTCGGCCAT   1800

CTGGCAGGTG GCTACGACGC CCAGTACTAC GGGTACCTGT GGAGCGAGGT GTATTCCATG   1860

GACATGTTCC ACACGCGCTT CAAGCAGGAG GGTGTCCTGA ACAGCAAGGT TGGCATGGAT   1920

TACAGAAGCT GCATCCTGAG ACCCGGCGGT TCCGAGGATG CCAGCGCCAT GCTGAGGCGC   1980

TTCCTGGGCC GTGACCCCAA GCAGGACGCC TTCCTCCTGA GCAΔGGGGCT GCAGGTCGGG   2040

GGCTGCGAGC CCGAGCCGCA GGTCTGCTGA   2070

(2) INFORMATION FOR SEQ ID NO:7:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 25 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

            TCGAATTCAA GGAGTACTTC CCTGT   25

(2) INFORMATION FOR SEQ ID NO:8:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 25 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: YES
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

            CAAACGTTTG GAAGGCCAGG CCCAG   25

(2) INFORMATION FOR SEQ ID NO:9:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 27 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

            CATGGCCTGC TGGGCATCTA CCAGGAG   27

(2) INFORMATION FOR SEQ ID NO:10:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 25 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single

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```
        (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: cDNA
  (iii) HYPOTHETICAL: NO
   (iv) ANTI-SENSE: NO
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

        TCGAATTCAA RGARTAYTTY CCNGT        25


(2) INFORMATION FOR SEQ ID NO:11:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH:  23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: cDNA
  (iii) HYPOTHETICAL: NO
   (iv) ANTI-SENSE: YES
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

        CAAAGCTTRT GRTTNGCNAG NCC        23


(2) INFORMATION FOR SEQ ID NO:12:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: cDNA
  (iii) HYPOTHETICAL: NO
   (iv) ANTI-SENSE: YES
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

        CAAAGCTTRT GRTTNGCYAA NCC        23


(2) INFORMATION FOR SEQ ID NO:13:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: cDNA
  (iii) HYPOTHETICAL: YES
   (iv) ANTI-SENSE: NO
   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

        AAGGAGTACT TCCCTGTGCA GGTGGTCACG CACGGGCTGC TGGCCATCTA CCAGGAGCTC        60

        CTGGGCCTGG CCTTC        75


(2) INFORMATION FOR SEQ ID NO:14:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 18 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: cDNA
  (iii) HYPOTHETICAL: NO
```

```
      (iv) ANTI-SENSE: NO
      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

          GAAATGCACG TGCCTGAG            18
```

```
(2) INFORMATION FOR SEQ ID NO:15:
      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 17 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: YES
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

          CCAGGACATA GTCGGCG             17
```

```
(2) INFORMATION FOR SEQ ID NO:16:
      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 164 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

  GAAATGCACG TGCCTGAGAC CAGGAGGAAA GTGGAGGAGG CCTTCAACTG CCGGTGCAAG    60

  CCGGTGCAAG GAGGAGAACT GCGCTATCCT CAAGGAGCTG GTGACGCTGC GGGCCCAGAA   120

  GTCCCGCCTG CTGGGGTTCC ACACGCACGC CGACTATGTC CTGG                    164
```

```
(2) INFORMATION FOR SEQ ID NO:17:
      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: YES
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

          ACTTTCCTCC TGGTCTCA            18
```

```
(2) INFORMATION FOR SEQ ID NO:18:
      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 18 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
```

(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

    GGAGAAGCTC ATTGAGTC   18

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We claim:

    1.   Human amyloidin protease, capable of cleaving the
Met-Asp bond in the substrate: acetyl-Ser-Glu-Val-Lys-
Met-Asp-Ala-Glu-Phe-Arg (Seq ID No:1), substantially free of
5   natural contaminants.

    2.   Human amyloidin protease, which has a molecular
weight of about 80,000 daltons as determined by SDS-
polyacrylamide gel electrophoresis.

    3.   The human amyloidin protease of claim 1, having the
10   primary sequence (Seq ID No: 5):

```
Met Lys Pro Pro Ala Ala Cys Ala Gly Asp Met Ala Asp Ala Ala Ser
1            5               10             15

Pro Cys Ser Val Val Asn Asp Leu Arg Trp Asp Leu Ser Ala Gln Gln
        20              25              30

Ile Glu Glu Arg Thr Arg Glu Leu Ile Glu Gln Thr Lys Arg Val Tyr
    35              40              45

Asp Gln Val Gly Thr Gln Glu Phe Glu Asp Val Ser Tyr Glu Ser Thr
    50              55              60

Leu Lys Ala Leu Ala Asp Val Glu Val Thr Tyr Thr Val Gln Arg Asn
65              70              75              80

Ile Leu Asp Phe Pro Gln His Val Ser Pro Ser Lys Asp Ile Arg Thr
            85              90              95

Ala Ser Thr Glu Ala Asp Lys Lys Leu Ser Glu Phe Asp Val Glu Met
            100             105             110

Ser Met Arg Glu Asp Val Tyr Gln Arg Ile Val Trp Leu Gln Glu Lys
    115             120             125

Val Gln Lys Asp Ser Leu Arg Pro Glu Ala Ala Arg Tyr Leu Glu Arg
    130             135             140

Leu Ile Lys Leu Gly Arg Arg Asn Gly Leu His Leu Pro Arg Glu Thr
145             150             155             160

Gln Glu Asn Ile Lys Arg Ile Lys Lys Leu Ser Leu Leu Cys Ile
            165             170             175

Asp Phe Asn Lys Asn Leu Asn Glu Asp Thr Thr Phe Leu Pro Phe Thr
            180             185             190

Leu Gln Glu Leu Gly Gly Leu Pro Glu Asp Phe Leu Asn Ser Leu Glu
            195             200             205
```

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Lys Met Glu Asp Gly Lys Leu Lys Val Thr Leu Lys Tyr Pro His Tyr
    210   215   220

5   Phe Pro Leu Leu Lys Lys Cys His Val Pro Glu Thr Arg Arg Lys Val
    225   230   235   240

Glu Glu Ala Phe Asn Cys Arg Cys Lys Glu Glu Asn Cys Ala Ile Leu
            245   250   255

10   Lys Glu Leu Val Thr Leu Arg Ala Gln Lys Ser Arg Leu Leu Gly Phe
            260   265   270

His Thr His Ala Asp Tyr Val Leu Glu Met Asn Met Ala Lys Thr Ser
            275   280   285

15   Gln Thr Val Ala Thr Phe Leu Asp Glu Leu Ala Gln Lys Leu Lys Pro
            290   295   300

Leu Gly Glu Gln Glu Arg Ala Val Ile Leu Glu Leu Lys Arg Ala Glu
20   305   310   315   320

Cys Glu Arg Arg Gly Leu Pro Phe Asp Gly Arg Ile Arg Ala Trp Asp
                325   330   335

25   Met Arg Tyr Tyr Met Asn Gln Val Glu Glu Thr Arg Tyr Cys Val Asp
                340   345   350

Gln Asn Leu Leu Lys Glu Tyr Phe Pro Val Gln Val Val Thr His Gly
                355   360   365

30   Leu Leu Gly Ile Tyr Gln Glu Leu Leu Gly Leu Ala Phe His His Glu
            370   375   380

Glu Gly Ala Ser Ala Trp His Glu Asp Val Arg Leu Tyr Thr Ala Arg
35   385   390   395   400

Asp Ala Ala Ser Gly Glu Val Val Gly Lys Phe Tyr Leu Asp Leu Tyr
                405   410   415

40   Pro Arg Glu Gly Lys Tyr Gly His Ala Ala Cys Phe Gly Leu Gln Pro
                420   425   430

Gly Cys Leu Arg Gln Asp Gly Ser Arg Gln Ile Ala Ile Ala Ala Met
            435   440   445

45   Val Ala Asn Phe Thr Lys Pro Thr Ala Asp Ala Pro Ser Leu Leu Gln
            450   455   460

His Asp Glu Val Glu Thr Tyr Phe His Glu Phe Gly His Val Met His
50   465   470   475   480

Gln Leu Cys Ser Gln Ala Glu Phe Ala Met Phe Ser Gly Thr His Val
            485   490   495

55   Glu Arg Asp Phe Val Glu Ala Pro Ser Gln Met Leu Glu Asn Trp Val
            500   505   510

```
     Trp Glu Gln Glu Pro Leu Leu Arg Met Ser Arg His Tyr Arg Thr Gly
             515                 520             525


     Ser Ala Val Pro Arg Glu Leu Leu Glu Lys Leu Ile Glu Ser Arg Gln
             530                 535             540


     Ala Asn Thr Gly Leu Phe Ser Leu Arg Gln Ile Val Leu Ala Lys Val
     545                 550             555                 560


     Asp Gln Ala Leu His Thr Gln Thr Asp Ala Asp Pro Ala Glu Glu Tyr
                 565             570             575


     Ala Arg Leu Cys Gln Glu Ile Leu Gly Val Pro Ala Thr Pro Gly Thr
             580                 585             590


     Asn Met Pro Ala Thr Phe Gly His Leu Ala Gly Gly Tyr Asp Ala Gln
             595                 600             605


     Tyr Tyr Gly Tyr Leu Trp Ser Glu Val Tyr Ser Met Asp Met Phe His
             610                 615             620


     Thr Arg Phe Lys Gln Glu Gly Val Leu Asn Ser Lys Val Gly Met Asp
     625                 630             635                 640


     Tyr Arg Ser Cys Ile Leu Arg Pro Gly Gly Ser Glu Asp Ala Ser Ala
                 645             650             655


     Met Leu Arg Arg Phe Leu Gly Arg Asp Pro Lys Gln Asp Ala Phe Leu
                 660             665             670


     Leu Ser Lys Gly Leu Gln Val Gly Gly Cys Glu Pro Glu Pro Gln Val
             675                 680             685


     Cys.
```

4.   The human amyloidin protease of claim 1, having an endopeptidic protease activity capable of cleaving the Asp-Ala and the Ala-Glu peptide bonds in the peptide.

5.   The human amyloidin protease of claim 1, which is a metalloprotease.

6.   A method for purifying human amyloidin protease from cells, said method comprising:
     a)   disrupting human cells to form an aqueous extract and an insoluble fraction;
     b)   using chromatographic fractionation on the aqueous extract to produce an enriched fraction having amyloidin protease activity;

c)   binding the enriched fraction of (b) to a hydrophobic
interaction chromatography matrix;

d)   eluting the bound protease activity by gradient
fractionation to form an eluate; and

e)   selecting for fractions from the eluate having the
ability to hydrolyze an internal Met-Asp peptide bond in an
amyloid-like peptide substrate.

7.   The method of claim 6, wherein the human cells are
present in brain tissue, and the brain tissue is homogenized
in the presence of a buffer having a pH of about 6 to 8.

8.   The method of claim 6, wherein the cells are
erythrocytes and cells disruption is by osmotic cell lysis.

9.   The method of claim 6, wherein the chromatographic
fractionation is selected from the group consisting of ion
exchange chromatography, dye ligand chromatography, size
exclusion chromatography, Chromatofocusing®, hydroxyapatite
chromatography or a combination thereof.

10.   The method of claim 9, wherein the ion exchange
chromatography employs an anion exchange resin for removal of
neutral or basic proteins in the aqueous extract to produce an
enriched fraction.

11.   The method of claim 9, wherein the enriched fraction
is applied to a hydrophobic interaction chromatography matrix
in a high salt buffer.

12.   The method of claim 11, wherein the high salt buffer
is approximately 1 M ammonium sulfate.

13.   The method of claim 11, wherein the hydrophobic
interaction chromatography matrix is a Phenyl-TSK HPLC column.

14. The method of claim 12, wherein gradient
fractionation of step (d) consists of a 1 to 0 M descending
gradient of ammonium sulfate.

5   15. The method of claim 14 which further comprises after
step (d), collecting in a pH 6 to 8 buffer, the amyloidin
protease.

16. The method of claim 9, wherein the chromatographic
fractionation is sequentionally performed using anion
exchange, hydroxyapatite and Chromatofocusing® chromatography.

10   17. The method of claim 9, wherein the chromatographic
fractionation is sequentionally performed using anion exchange
and Chromatofocusing® chromatography.

18. An antibody obtained by means of an immune response
in a mammal having been exposed to the human amyloidin
15   protease of claim 1.

19. An antibody obtained by means of an immune response
in a mammal having been exposed to a peptide fragment of the
protein of claim 1, said fragment corresponding substantially
to the sequence Val-Tyr-Asp-Gln-Val-Gly-Thr-Gln-Glu-Phe-
20   Glu-Asp-Val-Ser-Tyr-Glu-Ser-Thr-Leu-Lys (Seq ID No:2).

20. The antibody of claim 18, wherein the antibody is a
polyclonal antibody.

21. The antibody of claim 18, wherein the antibody is a
monoclonal antibody.

25   22. An immunogenic peptide fragment of the protease of
claim 1, wherein the peptide fragment is about 10 amino acids
in length.

23. The immunogenic peptide fragment of claim 22,
wherein the peptide fragment is about 20 amino acids.

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24. A method for the identification of an inhibitor of the protease of claim 1, said method comprising:

a) combining the prot ase with a compound of interest under conditions sufficient to form a mixture comprising a complex of the protease and the compound of interest;

b) introducing the mixture to an amyloid-like substrate under conditions sufficient to cleave the Met-Asp bond in said substrate; and

c) monitoring whether cleavage of the substrate has occurred.

25. The method of claim 24, wherein the protease and the compound of interest are initially combined in the presence of the substrate.

26. A method for the identification of an inhibitor of a protease capable of cleaving an endopeptidic Met-Asp bond in an amyloid-like substrate, said method comprising:

a) combining the protease with a compound of interest under conditions sufficient to cleave the Met-Asp bond of the amyloid-like substrate; and

b) monitoring whether cleavage of the substrate has occurred.

27. The method of claim 26, wherein the protease is human amyloidin protease.

28. The method of claim 26, wherein the protease is a mammalian chymase.

29. The method of claim 26, wherein the mammalian chymase is selected from the group consisting of human skin chymase and rat mast cell proteases I and II.

30. The method of claim 26, wherein the amyloid-like substrate corresponds substantially to the peptide:

acetyl-Ser-Glu-Val-Xaa$_4$-Xaa$_5$-Asp-Ala-Glu-Phe-Arg

wherein Xaa₄ is Gln or Lys and Xaa₅ is Met or Nle (Seq ID
No:1).

31.   The method of claim 26 wherein the amyloid-like
substrate is a fragment of the amyloid precursor protein.

5   32.   The method of claim 26, wherein the amyloid-like
substrate is labeled.

33.   The method of claim 32, wherein the label comprises
a signal means and a capture means.

34.   The method of claim 33, wherein the capture means is
10   a specific binding partner.

35.   The method of claim 33, wherein the signal means is
capable of emitting radiation.

36.   A DNA sequence encoding the human amyloidin protease
of claim 1.

15   37.   The DNA sequence of claim 36, comprising the
nucleotide sequence (Seq ID No:6):

```
     ATGAAGCCCC CCGCAGCCTG TGCAGGAGAC ATGGCGGACG CAGCCATCTCC GTGCTCTGTG        60

     GTAAACGACC TGCGGTGGGA CCTGAGTGCC CAGCAGATAG AGGAGCGCAC CAGGGAGCTC       120
20
     ATCGAGCAGA CCAAGCGCGT GTATGACCAG GTTGGCACCC AGGAGTTTGA GGACGTGTCC       180

     TACGAGAGCA CGCTCAAGGC GCTGGCCGAT GTGGAGGTCA CCTACACAGT TCAGAGGAAT       240

25   ATCCTTGACT TCCCCCAGCA TGTTTCCCCC TCCAAGGACA TCCGGACAGC CAGCACAGAG       300

     GCCGACAAGA AGCTCTCTGA GTTCGACGTG GAGATGAGCA TGAGGGAGGA CGTGTACCAG       360

     AGGATCGTGT GGCTCCAGGA GAAAGTTCAG AAGGACTCAC TGAGGCCCGA GGCTGCGCGG       420
30
     TACCTGGAGC GGCTAATCAA GCTGGGCCGG AGAAATGGGC TTCACCTCCC CAGAGAGACT       480

     CAGGAAAACA TCAAACGCAT CAAGAAGAAG CTGAGCCTTC TGTGCATCGA CTTCAACAAG       540

35   AACCTGAACG AGGACACGAC CTTCCTGCCC TTCACGCTCC AGGAGCTAGG AGGGCTCCCC       600

     GAGGACTTTC TGAACTCCCT GGAGAAGATG GAGGACGGCA AGTTGAAGGT CACCCTCAAG       660

     TACCCCCATT ACTTCCCCCT CCTGAAGAAA TGCCACGTGC CTGAGACCAG GAGGAAAGTG       720
```

```
GAGGAGGCCT TCAACTGCCG GTGCAAGGAG GAGAACTGCG CTATCCTCAA CGAGCTGGTG    780

ACGCTGCGGG CCCAGAAGTC CCGCCTGCTG GGGTTCCACA CGCACGCCGA CTATGTCCTG    840

GAGATGAACA TGGCCAAGAC CAGCCAGACC GTGGCCACCT TCCTAGATGA GCTGGCGCAG    900

AAGCTGAAGC CCCTGGGGGA GCAGGAGCGT GCGGTGATTC TGGAGCTGAA CCGTGCGGAG    960

TGCGAGCGCC GGGGCCTGCC CTTCGACGGC CGCATCCGTG CCTGGGACAT GCGCTACTAC   1020

ATGAACCAGG TGGAGGAGAC GCCGCTACTGC GTGGACCAGA ACCTGCTCAA CGAGTACTTC   1080

CCCGTGCAGG TGGTCACGCA CGGGCTGCTG GGCATCTACC AGGAGCTCCT GGGGCTGGCC   1140

TTCCACCACG AGGAGGGCGC CAGTGCCTGG CATGAGGACG TGCGGCTCTA CACCGCGAGG   1200

GACGCGGCCT CGGGGGAGGT GGTCGGCAAG TTCTACCTGG ACCTGTACCC GCGGGAAGGA   1260

AAGTACGGGC ACGCGGCCTG CTTTGGCCTG CAGCCCGGCT GCCTGCGGCA GGATGGGAGC   1320

CGCCAGATCG CCATCGCGGC CATGGTGGCC AACTTCACCA AGCCCACAGC CGACGCGCCC   1380

TCGCTGCTGC AGCATGACGA GGTGGAGACC TACTTCCATG AGTTTGGCCA CGTGATGCAC   1440

CAGCTCTGCT CCCAGGCGGA GTTCGCCATG TTCAGCGGGA CCCACGTGGA CGGGGACTTT   1500

GTGGAGGCGC CGTCGCAGAT GCTGGAGAAC TGGGTGTGGG AGCAGGAGCC GCTGCTGCGG   1560

ATGTCGCGGC ACTACCGCAC AGGCAGCGCC GTGCCCCGGG AGCTCCTGGA GCAGCTCATT   1620

GAGTCCCGGC AGGCCAACAC AGGCCTCTTC AGCCTGCGCC AGATCGTCCT CGCCAAGGTG   1680

GACCAGGCCC TGCACACGCA GACGGACGCA GACCCCGCCG AGGAGTATGC CGGCTCTGC    1740

CAGGAGATCC TCGGGGTCCC GGCCACGCCA GGAACCAACA TGCCTGCAAC CTTCGGCCAT   1800

CTGGCAGGTG GCTACGACGC CCAGTACTAC GGGTACCTGT GGAGCGAGGT GTATTCCATG   1860

GACATGTTCC ACACGCGCTT CAAGCAGGAG GGTGTCCTGA ACAGCAAGGT TGGCATGGAT   1920

TACAGAAGCT GCATCCTGAG ACCCGGCGGT TCCGAGGATG CCAGCGCCAT GCTGAGGCGC   1980

TTCCTGGGCC GTGACCCCAA GCAGGACGCC TTCCTCCTGA GCAAGGGGCT GCAGGTCGGG   2040

GGCTGCGAGC CCGAGCCGCA GGTCTGCTGA.                                   2070
```

38.　A cell transformed with a recombinant vector comprising the DNA sequence of claim 36, said transformed cell capable of producing human amyloidin protease.

FIG. 1

FIG. 2

3A　　　　　　　　　　　　　　3B

FIG. 3

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07290

I. CLASSIFICATION F SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 9/64, 15/57;C12Q 1/37; C07K 7/00, 15/28
U.S.Cl.: 435/23, 226, 252.3; 530/328, 326, 387; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
U.S.	435/23, 219, 226, 252.3; 530/328, 326, 387; 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

Computer Search - CA, Sequence and APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X,P Y	Biochem. Biophys. Res. Comm., Volume 174, Number 2, issued 31 January 1991, C.R. Abraham, et al., "A Calcium-Activated Protease From Alzheimer's Disease Brain Cleaves At The N-Terminus Of The Amyloid B-Protein", pages 790-796, see all.	1,5 — 18,20-27, 30-36, 38
X,P Y	J. Cell. Biochem., Volume supplement 15G, issued 06-25 April 1991, C.R. Abraham, et al., "Studies On The Proteolytic Degradation Of The Amyloid Precursor Protein In Alzheimer's Disease", page 115, see the abstract CH 024.	1,5 — 18,20-27, 30-36, 38
X Y	Neurobiology Of Aging, Volume 11, issued 08 May 1990, C.R. Abraham et al., "Studies On A Brain Protease That Cleaves At The N-Terminus Of B-Protein-Related Synthetic Peptides", page 303, see the abstract 207.	1-5 — 18,20-27, 30-36, 38

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 January 1992	19 MAR 1992
International Searching Authority	Signature of Authorized Officer
ISA/US	Charles Patterson

gp

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATI N CONTINUED FR M THE SEC ND SHEET

$\frac{X}{Y}$	Soc. Neuroscience Abst., Volume 15, issued 1989, C.R. Abraham, et al., "Proteolytic Processing of B-Protein Precursor-Related Synthetic Peptides", see page 648, the abstract 262.7.	1,5 _____ 18,20-27, 30-36, 38

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____ , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____ , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers _____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-17 and 22-35, drawn to a protease, method of preparation and method of use.
II. Claims 18-21, drawn to an antibody.
III. Claims 36-38, drawn to a DNA sequence.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
   telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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		(43) International Publication Date: 30 April 1992 (30.04.92)

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(30) Priority data:
594,122   5 October 1990 (05.10.90)   US
766,351   30 September 1991 (30.09.91) US

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(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: AMYLOIDIN PROTEASE AND USES THEREOF

(57) Abstract

A proteolytic enzyme isolated from human tissue which exhibits a proteolytic activity to hydrolyze Met-Asp peptide bond in an amyloid-like substrate is disclosed. This enzyme has been designated "amyloidin" because it proteolytically cleaves a Met-Asp bond similar to the one present in the amyloid procursor protein to release a fragment having the mature Asp terminus of the β-amyloid peptide. Antibodies to the amyloidin protease is also provided. Methods to isolate and purify the amyloidin protease is provided, as well as assays to screen for inhibitors of the amyloidin protease. Also disclosed is the gene encoding the protease and methods for expression of the protease by recombinant DNA means.

* (Referred to in PCT Gazette No. 19/1992, Section II)

5

AMYLOIDIN PROTEASE AND USES THEREOF

Field of the Invention

The present invention relates to purification of
mammalian enzymes and more particularly to the purification of
10   human amyloidin protease, the identification of the gene
encoding the protease, the identification of inhibitors of
this protease, and various uses thereof.

Background of the Invention

Proteases are enzymes possessing the activity of
15   hydrolyzing peptide bonds in proteins and polypeptides. One
subclass of proteases, the metalloproteases are dependent on
an integral zinc atom catalysis and often require exogenous
calcium for activity. One such enzyme, which has been
referenced in the literature as collagenase-like peptidase (EC
20   3.4.99.31), Pz-peptidase (Barrett (1990) Biol Chem Hoppe-
Seyler 371(Supp):311-320) or metalloendopeptidase (EC
3.4.24.15) (Orlowski, et al (1989) Biochem J 261:951-958)
cleaves preferentially bonds on the carboxyl side of
hydrophobic amino acid residues and is believed to function in
25   the metabolism of bioactive peptides.

Similar enzymatic activity towards collagen sequence-
based peptides have been detected in a number of human tissue
extracts by various investigators; however, most of the work
was confined to the measurement of peptidase activity using
30   collagen sequence-based peptides (Lessley, et al (1985) J
Androl 6(6):372-378; Rajabi, et al (1984) Am J Obstet Gynecol
150(7):821-826 and Ito, et al (1977) Clin Chim Acta 78(2):267-
270). Pierotti, et al (1990) Biochem 29:10323-10329 recently
report the molecular cloning and primary structure of rat

testes metalloendopeptidase. The enzyme is composed of 645
amino acids with a molecular weight of 72,985 daltons. There
does not appear to be any reports that provide identification
of human Pz-peptidase, either by partial purification and

5   characterization, or by using a battery of substrates or
inhibitor profiles.

Recently, investigators have preliminarily identified a
proteolytic activity from human brain of about 68,000 daltons
that is capable of cleaving between the Met and Asp residues

10   of a small synthetic peptide HSWVKMDAEF, which corresponds to
amino acids 592 through 600 in the ß-amyloid precursor protein
("APP") (Abraham, et al (1990) Neurobiol Aging 11A:303) with
an N-terminal His. When this peptide, having an ^{125}I
radioiodinated His residue, is incubated with "brain protease"

15   fractions, fragments are generated and separated by thin layer
chromatography (TLC). The N-terminal fragments were detected
by exposure of the TLC plate to film (Abraham, et al (1991)
Biochem Biophys Res Comm. 174: 790-796). The cleavage pattern
obtained with the brain protease preparation was primarily at

20   three sites, between the Lys-Met, Met-Asp, and Asp-Ala, with
some cleavage obtained at His-Ser. The peptide cleavage was
inhibited by diisopropylfluorophosphate (DFP), α_1-
antichymotrypsin, and protease nexin II, all of which only
inhibit serine proteases. Based on chemical crosslinking

25   studies with the iodinated peptide substrate, two bands, one
at approximately 68,000 daltons and another at approximately
30,000 daltons, are suggested to be candidates for the
protease(s) in the preparation.

In more recent presentations (Abraham, et al (1991) J
30   Cell Biochem Suppl. 15G:115; Abraham, et al (1991) J Neurochem
57 (Suppl.):5109), these investigators claim at least two
different proteases in the preparation, one being the
previously described calcium-dependent serine protease, and
the other a cysteine metalloprotease. To date, no structure
35   or characterization or any of these proteases has been
presented.

APP is a membrane-spanning glycoprotein that is expressed
in many mammalian tissues and cell lines and is encoded by a

gene that, in humans, is found on chromosome 21. The
ß-amyloid core protein, referred to as the ß- or A4 peptide,
is an approximately 39-42 amino acid long peptide fragment of
APP, and is the major component of the myriad amyloid deposits
5   that accumulate extracellularly in the brains of patients with
Alzheimer's disease (AD) or form the cerebrovascular amyloid
in associated blood vessels.

There are at least three forms of the precursor protein:
APP695 (Kang, et al (1987) Nature 325:733); APP751 (Ponte, et
10   al (1988) Nature 331:525); and APP770 (Kitaguchi, et al (1988)
Nature 331:530) which refer to the number of amino acids in
the primary protein transcript. All of these forms contain
the ß-peptide sequence, which starts 28 amino acids N-terminal
to the beginning of the putative transmembrane region, and
15   ends approximately 14 amino acids in the transmembrane region.
The numbering of amino acids as used herein corresponds to
that used for APP695.

Recent work on the metabolism of the APP in cell culture
has clearly shown that after intracellular maturation of the
20   full transmembrane form of the protein, there is a specific
proteolytic processing event which leads to extracellular
secretion of a large N-terminal region, and leaves behind in
cell membranes a small, C-terminal fragment reactive with
antisera to the carboxyl end of the APP (Oltersdorf, et al
25   (1990) J Biol Chem 265:4492). The size of this C-terminally
reactive fragment made it likely that it contains the entire
ß-peptide. However, characterization by direct protein
sequencing of the N-terminal of this fragment showed that it
starts at Leu17 of the ß-amyloid core peptide where Asp597 of
30   APP695 is counted as Asp1 of the ß-peptide (Esch, et al (1990)
Science 248:1122). Characterization of the soluble secreted
form by isolation of the peptide containing its C-terminal
region also clearly showed that it ends at Gln15. Thus, in
this normal processing pathway, the transmembrane form of APP
35   is cleaved inside the ß-peptide (either before or after Lys16,
which is missing; presumably, it is taken off either by a
carboxypeptidase or an aminopeptidase activity post-cleavage),
and thus this pathway precludes the formation or deposition of

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the ß-peptide. It also follows then that an alternative
proteolytic pathway must exist for generation of the
ß-peptide.

5   The most likely characteristic of such a pathway would be
a proteolytic cleavage between Met596 and Asp597, since
protein sequencing of either senile (core) or vascular amyloid
always starts at this aspartic acid residue, although there
has been reported to be a ragged N-terminus for core amyloid
(Masters, et al (1985) <u>Proc Natl Acad Sci USA 82</u>:4245). The
10   preliminary work reported by Abraham et al (1990) supra,
provides some insight as to how APP might be proteolytically
processed to release the ß-amyloid core protein.

        Identification of mammalian proteases that are capable of
cleavage at this site is essential in order to screen for
15   inhibitors of such cleavage. Such inhibitors would be useful
for therapeutic intervention in AD.

        Cell culture models of the blood brain barrier may be
used for the design of drug delivery systems for the
inhibitors of the present invention. Such cell culture models
20   are disclosed in PCT/US90/05106, filed 13 September 1990 and
PCT/US90/05105, filed 13 September 1990.

<u>Disclosure of the Invention</u>

        The present invention provides human amyloidin protease,
capable of cleaving the Met-Asp peptide bond in the peptide
25   N-acetyl-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg (Seq ID
No:1), substantially free of natural contaminants. The
protease has an apparent molecular weight in the range of
about 80,000 daltons as determined by SDS-polyacrylamide gel
electrophoresis (under both reducing and nonreducing
30   conditions).

        Methods for the purification of amyloidin protease from
human cells, including blood and brain tissue, are also
provided. This protease may be used as a reagent in methods
for the identification of inhibitors against this prot ase.
35   Such methods include combining the protease with a putative
inhibitor in the presence of an amyloid-like substrate under
conditions suffici nt to cleave the Met-Asp bond; and

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monitoring the reaction to see whether cleavage of the
substrate has occurred.

     As an additional aspect of the invention, the amyloidin
protease can be used to raise antibodies, using either another
5   species of animal, such as a rabbit, or a hybridoma cell line.
The resulting antibodies are specific for the amyloidin
protease and can be used in diagnostic tests such as, for
example, an immunoassay, or in immunopurification methods.

     Further, it has been found that antigenic potential
10 resides in fragments of the whole amyloidin molecule. Thus,
it is possible to raise antibodies that specifically recogniz
an immunogenic epitope of an amyloidin protease using a
fragment of the polypeptide. The resulting antibodies can
themselves be used for immunopurification of the respective
15 protease or in diagnostic assays.

     Also provided as an aspect of the invention is the gene
encoding the human amyloidin protease, vectors containing the
gene and host cells transformed with the gene which are
capable of expressing human amyloidin protease.

20 **Brief Description of the Drawings**

     FIG. 1 is a chromatogram of amyloidin taken after the
phenyl-TSK chromatography step. The characteristic three-site
cleavage of N-acetyl-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg
(Seq ID No:1) by the eluted enzyme is shown.
25   FIG. 2 is a Coomassie-stained gel of purified amyloidin.

     FIG. 3A and 3B are Western blot analyses of rabbit
polyclonal antisera against an amyloidin protease synthetic
peptide.

     FIG. 4 is a Western blot analysis of a number of
30 monoclonal antibodies against the amyloidin protease. RAB =
rabbit polyclonal antisera.

Modes of Carrying Out the Invention

A.   **Definitions**

     "Amyloidin protease" as used herein refers to a native,
35 human proteolytic enzyme which shares some homology to Pz-
peptidase from other mammalian sources. The term also

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includes synthetic human amyloidin proteases, i.e., proteins produced by recombinant DNA means, dir ct chemical synth sis or a combination of both. Amyloidin protease is a polypeptide found, _inter alia_, in brain tissue and in blood.

5   The "amyloidin protease activity" of a protein refers to a peptide hydrolysis activity selective for a Met-Asp peptide bond similar to that found at the junction separating the ß-amyloid core peptide from the amino-terminal region of APP. This activity can be assayed _in vitro_ by incubating the
10   amyloid protease with a synthetic substrate corresponding to the peptide sequence including the Met-Asp junction and determining the extent of cleavage. The amyloid protease activity predominantly cleaves the Met-Asp bond, although additional cleavage of certain amino-terminal ß-amyloid core
15   peptide residues is observed with at least one of the amyloid proteases of the invention. This multiple cleavage activity may contribute to the formation of the ragged amino-terminus of the ß-amyloid core peptide originally observed by Masters, et al (1985), supra.

20   As used herein, "amyloid-like substrate" refers to an "amyloidogenic" polypeptide derived from the APP which has substantial homology to the region of the APP spanning the peptide sequence at the Met-Asp bond located at the amino-terminus of the ß-amyloid core peptide. The source of
25   the polypeptide includes, but is not limited to, microbially expressed APP or fragments thereof containing the Met-Asp cleavage site, endogenous APP present in biological materials such as cells or mammalian tissue homogenates, and synthetically produced peptides.

30   A peptide "derived from" a designated polypeptide sequence refers to a sequence which is comprised of a sequence of at least 6 amino acids, and preferably at least about 10-12 amino acids corresponding to a region of the designated polypeptide sequence. "Corresponding" means identical to or
35   exhibiting a minimum of about 60% or more amino acid identity with the designated sequence. The derived sequence is not necessarily physically deriv d from the polypeptide sequence but may be generated in any mann r, including chemical

synthesis or DNA replication of the gene encoding the polypeptide and microbial expression thereof.

B.   General Method

The practice of the present invention will employ, unless
5   otherwise indicated, conventional techniques of protein
purification, molecular biology, microbiology and recombinant
DNA techniques, which are within the skill of the art.   Such
techniques are explained fully in the literature.   See, e.g.,
"Guide to Protein Purification" in Methods in Enzymology (M.P.
10   Deutscher, ed., (1990) Academic Press, Inc.); Sambrook,
Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual,
Second Edition (1989); Oligonucleotide Synthesis (M.J. Gait,
ed., 1984); Nucleic Acid Hybridization (B.D. Hames & S.J.
Higgins, eds., 1984); A Practical Guide to Molecular Cloning
15   (B. Perbal, 1984); PCR Protocols, A Guide to Methods and
Applications (M.A. Innis, et al, eds., (1990) Academic Press,
Inc.); Current Protocols in Molecular Biology (F.M. Ausubel,
et al, eds., (1989) John Wiley & Sons); and additional
publications in the series, Methods in Enzymology (Academic
20   Press, Inc.). All patents, patent applications, and
publications mentioned herein, both supra and infra, are
hereby incorporated by reference.

The present invention provides substantially purified
human amyloidin protease free from natural contaminants.
25   Purified amyloidin protease allows for the amino acid sequence
to be determined, nucleic acid probes designed and amyloidin
protease genes to be cloned.   Once cloned, the amyloidin
protease gene can be used to produce recombinant amyloidin
protease.

30   The identity and characterization of human amyloidin
protease further permits the development of in vitro screening
models for agents which inhibit the cleavage of the Met-Asp
bond in APP.   If such cleavage inhibition is successful, β-
amyloid core protein formation is prevented.   Thus, this mod l
35   provides a new and valuable medium with which to explore the
molecular pathog n sis of amyloidosis rel vant to AD and to
evaluate potentially therapeutic agents.

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As part of the initial work to identity proteases having proteolytic specificity for the Met-Asp peptide bond of APP, the following synthetic decapeptide was designed: N-acetyl-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg. The
5   C-terminal end can be either a free carboxylate (COOH) or an amide (CO-NH,).

This peptide spans the putative Met-Asp cleavage site and is referred to herein as APP592-601. In addition to this substrate, other amyloid-like substrates may be employed in an
10   inhibition assay to screen for protease activities isolated from mammalian sources which are capable of cleaving the Met-Asp peptide bond in this sequence. As taught in the examples, amyloid-like substrates also may be used to characterize the substrate specificity of the amyloid
15   proteases described in the present invention. While endopeptidic cleavage predominates at the Met-Asp bond, cleavage may also occur at the Asp-Ala and Ala-Glu bonds of the β-peptide. Endopeptidic cleavage of the amyloid-like substrate is detected by reverse phase high performance liquid
20   chromatography (HPLC), and the site of cleavage determined by amino acid analysis of the peptide fragments.

To isolate the amyloid protease activities of the present invention, extracts of mammalian tissues were made in a variety of aqueous buffers, such as, for example, Tris,
25   phosphate and HEPES, of about 20-50 mM pH 7.5, at 4-8°C, using conventional homogenation procedures, such as, for example, Waring blender or Teflon homogenizer, followed by repeated centrifugation at 10,000-15,000 x g. The supernatant soluble fraction from this stage ("low ionic strength") are
30   decanted and the pellet is further extracted with high ionic strength salt, for example 1 M NaCl, and a detergent, such as 1% Triton X-100. If, however, the amyloidin proteases are to be isolated from mammalian cells such as erythrocytes, conventional cell disruption techniques may be employed. Such
35   techniques include homogenization, sonication, osmotic lysis and pressure cycling. Prior to disruption, the cells may be first conc ntrated by filtration, centrifugation, or other conventional methods.

The various extracts are generally incubated with the
amyloid-like substrates under a variety of conditions. For an
initial screen, about 25 μl of the extract are incubated with
10 μl of a 2 mg/ml solution of the amyloid-like substrate,
5   along with 10 μl of 1 M Tris-HCl, pH 7.5, and 5 μl of a
solution of water, or 10 to 100 mM CaCl, or 10 to 100 mM EDTA.
After a 30-60 min incubation of the reaction mixture in a
water bath at 37°C, the reactions are quenched by addition of
multiple volumes of ice-cold ethanol, placed in ice for about
10   20 min and the samples are centrifuged at 15,000 x g for 10
min in an Eppendorf microfuge. The supernatant is removed,
dried under vacuum evaporation and reconstituted with water.

Aliquots of the sample are analyzed by injection onto a
C18 reversed-phase HPLC column and elution with a 0-60%
15   gradient of 0.1% TFA/acetonitrile, to assess the degradation
of the substrate. Multiple fragments were produced when the
peptide was incubated in the presence of from 0.1 to 10 mM
Ca^{++} ions at a pH range over 6.5 to 8, with soluble fractions
obtained from low-ionic strength extracts. Little peptide
20   cleavage activity was detected under other extraction
conditions, for example, with high salt or detergents.
Analysis of the peptide fragments produced by quantitative
amino acid analysis indicated that the major sites of cleavage
were between the Met-Asp, the Asp-Ala, and the Ala-Glu bonds.
25   The Met-Asp cleaving activity from the crude extract was
isolated for further purification.

The purification methods referred to herein include a
variety of procedures. Among several types which may be
useful are size fractionation using molecular sieve
30   chromatography; ion exchange chromatography under suitable
conditions; adsorption chromatography using nonspecific
supports, such as hydroxyapatite, silica, alumina, and so
forth; dye-ligand interaction chromatography, such as Cibacron
Blue F3GA-Sepharose, Chromatofocusing®; and also gel-supported
35   electrophoresis. In th case of the amyloidin protease,
hydrophobic interaction chromatography, such as using
phenyl-Sepharose, phenyl-Superose or phenyl-TSK, has been
shown to be particularly useful to separate the amyloid

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protease activity from natural contaminants including an
activity which, while not consistently reproducible, shares
the Met-Asp cleavage activity. Hydrophobic interaction
chromatography also serves to provide substantial
5   purification. This procedure separates proteins based on the
hydrophobic properties of the protein, unlike ion exchange
chromatography which separates based on charge properties of
the protein.

     In addition, initial purification of the proteases using
10   ion exchange chromatography (such as with using weak anion
exchangers, for example, DEAE-Sepharose) has been shown to be
a particularly effective procedure to increase the purity of
the amyloidin protease. While the ion exchange chromatography
process is described herein primarily with respect to a
15   cross-linked cellulose having functional diethylaminoethyl
moieties and sold, for example, under the trademark DE52
(Whatman), other resins, particularly other mildly anionic
resins, are suitable for partially purifying amyloidin
protease-containing extracts by ion exchange chromatography.
20   Other suitable resins include but are not limited to
cross-linked dextran having DEAE moieties, and polystyrene
cross-linked with benzene having polyaminoethylene moieties.
When the amyloidin protease is being prepared bacterially or
in some other culture, as will be possible using recombinant
25   DNA procedures, pre-purification steps may be omitted.

     Each of these purification techniques are, in a general
sense, well known in the art, and a detailed description of
the peculiarities of their specific application to the
amyloidin protease is described in the examples below.
30   During the isolation steps, purification of the amyloidin
protease is monitored by testing chromatography fractions for
its ability to cleave the Met-Asp peptide bond in an
amyloid-like substrate as analyzed by RP-HPLC.

     Amyloidin has multiple cleavage sites, although the
35   predominant cleavage site occurs at the Met-Asp peptide bond.
Replacement of amino acid residues at th amino-terminal
region of the amyloid-like substrate can liminate or reduc
cleavage at the additional sites as shown in Example 5 herein.

Once substantially purified, the native amyloidin
protease may also be subjected to amino acid sequence
analysis. Applying conventional peptide sequencing
procedures, using for example, an Applied Biosystems model
5   470A gas-phase sequencer amino acid sequences for each of the
amyloidin proteases may be generated.
The amino acid composition of amyloidin is as follows:

	Residue	Predicted Composition*
	Asx	58.6
10	Glx	94.2
	Ser	31.7
	Gly	51.7
	His	23.6
	Arg	61.0
15	Thr	32.8
	Ala	57.1
	Pro	29.1
	Tyr	26.0
	Val	43.1
20	Met	16.3
	Cys	8.6
	Ile	18.7
	Leu	83.3
	Phe	30.0
25	Trp	ND
	Lys	44.1

*Predicted Composition equals the number of
approximate amino acids; Asx and Glx refer to
(Asp and Asn) and (Glu and Gln), respectively;
30   Cys was not quantitatively determined; Trp
was not determined.

The purified amyloidin protease can be used to raise
either polyclonal or monoclonal antibodies. The amyloidin
protease is injected into a mammal, such as rabbits, mice or
35   guinea pigs, and the resulting antibodies recovered from the
serum. Alternatively, monoclonal antibodies may be produced
by immunization of mice with either the purified protein or
fragments thereof, and fusion of their splenic cells with
murine myeloma or plasmacytoma cells. These protocols are
40   conventional in the art.
One of the internal peptides of amyloidin has been shown
to be immunogenic. This sequence, as well as other
immunogenic regions, may be produced synthetically using

available amino acid synthesizers. Such immunogenic peptides contain epitopes, that is, a determinant responsible for specific interaction with an antibody molecule.

Antibodies to either the whole amyloidin protease or to
5   immunogenic fragments derived therefrom can be used in standardized immunoassays, such as radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA). In addition, such antiboies may be used to localize the protease in immunochemical or immunohistochemical methods, as further
10   described in the examples.

Once the amino acid sequence is determined, recombinant DNA encoding the amyloidin protease may be prepared. First, oligonucleotide probes encoding a portion of the determined amino acid sequence are prepared and used to screen DNA
15   libraries for the gene encoding the amyloidin protease. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those cf ordinary skill in the art. See, for example, DNA Cloning: Volume I (D.M.
20   Glover, ed. 1985); Nucleic Acid Hybridization, supra; Current Protocols in Molecular Biology, supra; and Molecular Cloning: A Laboratory Manual, supra.

First, a DNA library is prepared. The library can consist of a genomic DNA library from a selected mammal, such
25   as human. DNA libraries can also be constructed of cDNA prepared from a poly-A RNA (mRNA) fraction by reverse transcription. The mRNA is isolated from a cell line or tissue known to express the amyloidin protease. cDNA (or genomic DNA) is cloned into a vector suitable for construction
30   of a library. A preferred vector is a bacteriophage vector, such as phage lambda. The construction of an appropriate library is within the skill of the art. Alternatively, the cDNA or genomic library may also be purchased from commercial sources, for example, Clontech and Stratagene, Inc.
35   Once the library is obtained, oligonucleotides to probe the library are prepared and used to isolate the desired amyloidin gene. The particular nucleotide sequences s lect d are chosen so as to correspond to the codons encoding a known

amino acid sequence from the amyloidin protease. Since the
genetic code is redundant, it will often be nec ssary to
synthesize several oligonucleotides to cover all, or a
reasonable number, of the possible nucleotide sequences which

5   encode a particular region of the protein. One can also
design a single probe or "guessmer" wherein one uses codon
bias and other considerations, such as CG dinucleotide
underrepresentations to guess the best sequence, or by using
inosine bases where ambiguity in the sequence exists

10   (Sambrook, et al, supra). It may also be desirable to use two
probes (or sets of probes), each to different regions of the
gene, in a single hybridization experiment. Automated
oligonucleotide synthesis has made the preparation of probes
relatively straightforward. In addition, probes may be

15   commercially obtained.

        Alternatively, one may use the polymerase chain reaction
(PCR) to amplify a portion of the desired gene encoding the
amyloidin protease. In its simplest form, PCR is an _in vitro_
method for the enzymatic synthesis and amplification of

20   specific DNA sequences, using two oligonucleotide primers that
hybridize to opposite strands and flank the region of interest
in the target DNA. A repetitive series of cycles involving
template denaturation, primer annealing, and the extension of
the annealed primers by DNA polymerase results in the

25   exponential accumulation of a specific fragment whose termini
are defined by the 5' ends of the primers. PCR reportedly is
capable of producing a selective enrichment of a specific DNA
sequence by a factor of $10^{\bullet}$. The PCR method is described in
Saiki, et al (1985) _Science 230_:1350 and is the subject of

30   U.S. Patent Nos. 4,683,195, 4,683,202 and 4,800,159. The
portion of the amyloidin gene synthesized by the PCR technique
will be used to probe cDNA libraries for clones encoding the
full length amyloidin cDNA.

        Because the genetic code is redundant, PCR from known

35   amino acid sequence requires PCR with eith r degenerate,
inosine substituted, or "guessmer" PCR oligos. (See PCR
Protocols, Innis, et al, supra, especially the chapter on
"Degenerate Primers for DNA Amplification" at pp. 39-45;

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Sambrook, et al, supra; Maisonpiere, et al (1990), <u>Science</u>
<u>247</u>:1446; Hohn, et al (1990) <u>Nature 344</u>:339. These
techniques have been widely used to clone a variety of genes
as described in the above references. One can use PCR to
amplify DNA sequences from either cDNA generated from RNA,
genomic DNA or from a cDNA or genomic library. Strategies
using either conventional PCR as described above, or "anchor"
PCR could be used. In anchor PCR, one uses a library
containing the amyloidin gene as the PCR substrate, and uses
one sequence within the amyloidin gene and another within the
vector that the library is in so that the region amplified
contains sequences from the vector as well as from the
amyloidin gene. In this case only very limited amino acid
information is necessary. PCR conditions and components such
as temperatures, concentrations of magnesium, <u>Taq</u> polymerase,
and oligos would be optimized as described in Innis, et al,
supra. One might also utilize conditions where 7-deazaguanine
is used to allow the amplification of sequences containing
secondary structure.

   As an alternative to cloning the gene based on nucleic
acid probes, one can use the amino acid sequence of amyloidin
to prepare antibody probes that can be used to screen for the
amyloidin gene. Given the amino acid sequence, peptides of
identical sequence can by synthesized by standard techniques,
and these peptides can be used to immunize rabbits or mice.
Polyclonal or monoclonal antibodies to either amyloidin or
peptides derived therefrom can be generated and used to detect
amyloidin clones from an appropriate library. Libraries made
in vectors which are designed to express the gene of interest,
include but not limited to lambda gt11, lambdaZAP, or
lambdaORF8 (see Ausubel, et al, supra, and Sambrook, et al,
supra) can be screened with an antibody to that library.
Libraries in these vectors can be generated or purchased from
sources such as Clontech or Stratagene. The protein of the
cloned gene is expressed in these vectors and the ability of
an antibody to bind to the expressed protein allows one to
identify the amyloidin clone by standard antibody probing
techniques.

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A DNA molecule containing the coding sequence for amyloidin protease can be cloned in any suitable vector and thereby maintained in a composition substantially free of vectors that contain the coding sequence of other mammalian
5   genes, including those encoding other amyloidin protease activities. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice.

For expression of the amyloidin protease, a variety of
10 . systems can be used, including, but not limited to, bacterial, yeast, insect, and mammalian systems.

Bacterial expression vectors such as pEx12Mcr and pEx10mer (Seedorf, et al (1987) EMBO J 6:139) and a variety of vectors discussed in the above cited publications, can be used
15   to express fusion proteins that contain amyloidin sequences linked to bacterial genes. Other bacterial expression vectors can be used to make intact full-length amyloidin in bacterial cells. For bacterial expression the vector needs to have a bacterial promoter and a ribosome binding site.

20   Mammalian vectors useful in the present invention include, but are not limited to pORFex13 (Bernard, et al (1987) EMBO J 6:133), pL1, pcDV1, pcD-X (all from Okayama and Berg (1983) Mol Cell Biol 3:280), pSV2 and derivatives thereof including pSVneo and pSVdhfr (Sambrook, et al, supra), pRSVneo
25   (Gorman, et al (1983) Science 221:551 and vectors derived from these and/or related vectors such as pRSVcat (Gorman, et al (1982) Proc Natl Acad Sci USA 79:6777) can be used to express amyloidin in a variety of mammalian cell types. The amyloidin gene is placed in these vectors in operable juxtaposition and
30   then put into animal cells by standard techniques. Once inside the cell the protein is expressed from these vectors containing the amyloidin gene. Such expression control elements for expression in animal cells include a promoter, enhancer, splice site (this is optional) and polyadenylation
35   sequences. A variety of systems are available for expr ssion in animal virus systems, such as, for example, bovine papiloma virus, retroviruses, SV40 and other virus s as describ d in Ausubel, et al, supra and Sambrook, et al, supra, and in "High

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Level Production of Proteins in Mammalian Cells" by Randal J.
Kaufman, in _Genetic Engineering_ (1987) vol 9:155-198, Jane K.
Stelow, ed.

5   The insect virus system based on baculovirus vectors can
also be used to express the amyloidin gene. Insect virus
systems are commercially available from Invitrogen Corp., San
Diego, CA. Such vectors include, but are not limited to
pAc373 ("A Manual for Methods for Baculovirus Vectors and
Insect Cell Culture Procedures" by Max Summers and Gale Smith,
10   published by Texas Agricultural Experiment Station), pVL941
(Luckow and Summers, (1989) _Virology 170_:31). These vectors
can be used to transfer the amyloidin gene into a baculovirus
and the recombinant virus thus obtained used to infect insect
cells. The infected insect cells are used to produce
15   amyloidin protein. Complete methods for these procedures are
described in Summers, et al, supra.

       The amyloidin protease of the present invention can be
used to develop and/or identify agents which inhibit the
cleavage of the Met-Asp bond similar to that found in APP.
20   The selection of appropriate inhibitor molecules will
generally be guided by the rate at which the test compound
inhibits cleavage of the amyloid-like substrate. Inhibition
assays may be developed to assess the inhibitory spectrum of
various test compounds on the cleavage of the amyloid-like
25   substrate in the presence of the amyloidin protease.

       A suitably modified amyloid-like substrate may be
incubated, under conditions of neutral pH in a suitable
aqueous buffer, with a protease that has been incubated with a
potential inhibitory compound (at room temperature for 15-30
30   min, for example), for 1-4 hr, or a period of time sufficient
to obtain significant endopeptidic cleavage of the substrate
in the absence of the inhibitory agent. The proportion of
cleavage is then quantitated. Suitably varying the
concentration of the inhibitor compound and measuring the
35   inhibition of cleavage as compared to zero inhibitor
concentration, will allow one to determin an inhibition
curve, from which the inhibitory efficacy, such as the
inhibitor conc ntration at which 50% of th enzyme's cleavage

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activity is inhibited, or the inhibition constant (Ki) can be calculated by standard methods.

The present invention also provides the discovery that "clipsin", a chymotrypsin-like protease, selectively cleaves the amyloid substrate APP592-601 at the Met-Asp peptide bond. Clipsin was first reported by Nelson and Siman (1990) _J Biol Chem 265_:3836, and was partially purified from rat brain. These investigators showed that clipsin was relatively specific for the APP, but they did not identify any specific cleavage site. Also reported was the specificity of additional known proteins, such as calpain, for APP.

As shown herein, selectivity for the Met-Asp peptide bond persists even when clipsin is subjected to a further purification step (chromatography on a soybean trypsin inhibitor affinity column). These data are not obvious in view of the strong Suc-Ala-Ala-Pro-Phe-pNa (K_{cat}/K_m = 57,000) and weak Suc-Ala-Ala-Pro-Met-pNA (K_{cat}/K_m = 5,200) cleavage activity of clipsin previously reported. While the Nelson and Siman paper dismissed the possibility that clipsin might be one of the identified rat mast cell proteases, RMCP I and II (Woodbury, et al (1981) _Methods in Enzymol 80_:588), comparison of the enzymatic properties of clipsin with authentic RMCP I and with human skin chymase (the analogous human mast cell enzyme disclosed in Schechter, et al (1986) _J Immunol 137_:962) using the amyloid-like substrate APP592-601, clearly showed that all three proteases cleaved the APP592-601 peptide at the Met-Asp bond. Nelson, et al (1990) _Soc Neuroscience Abstr 16_:788) have recently reported more complete purification of "clipsin", including the partial amino acid sequence from the N-terminus, which indicate that clipsin indeed is RMCP I.

RMCP I, RMCP II and human skin chymase are known to belong to a family of related chymotrypsin-like proteases, also called "chymases." Members of this family include, for example, mouse mast cell proteases 1-6 (Reynolds, et al (1990) _Proc Natl Acad Sci USA 87_:3230-3234) and possibly, human cathepsin G. Each of these mammalian prot as s which exhibit the Met-Asp cleavage activity may be consider d equivalents

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for purposes of testing inhibitory agents of the amyloidin
protease of the present invention.

For analysis of cleavage inhibition, the amyloid-like
substrate may be labeled, as described below, by incorporating

5   moieties detectable by spectroscopic, photochemical,
biochemical, immunochemical, or chemical means. The method of
linking or conjugating the label to the amyloid-like substrate
depends, of course, on the type of label(s) used and the
position of the label on the substrate.

10   A variety of labels which would be appropriate for use in
the invention, as well as methods for their inclusion in the
substrate, are known in the art and include, but are not
limited to, enzymes (e.g., alkaline phosphatase and
horseradish peroxidase) and enzyme substrates, radioactive

15   atoms, fluorescent dyes, chromophores, chemiluminescent
labels, ligands having specific binding partners, or any other
labels that may interact with each other to enhance, alter, or
diminish a signal.

"Specific binding partner" refers to a protein capable of

20   binding a ligand molecule with high specificity, as for
example in the case of an antigen and a monoclonal antibody
specific therefor. These types of binding partners are also
referred to in the art as "capture" labels. Other specific
binding partners include biotin and avidin or streptavidin,

25   IgG and protein A, and the numerous receptor-ligand couples
known in the art. It should be understood that the above
description is not meant to categorize the various labels into
distinct classes, as the same label may serve in several
different modes. For example, ^{125}I may serve as a radioactive

30   label or as an electron-dense reagent. HRP may serve as
enzyme or as antigen for a monoclonal antibody.

Further, one may combine various labels for a desired
effect. In some situations it may be desirable to use two
labels on a single substrate with due consideration given for

35   maintaining an appropriate spacing of the labels to permit the
separation of the labels during hydrolysis of the peptide
bond. For example, one might label a substrate at its
N-terminus with biotin and its C-terminus with a radioactive

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label. One would detect cleavage of the substrate by passing
the reaction mixture over or through a solid phase extractant
(SPE) containing avidin or streptavidin. The SPE is monitored
to assess whether the signal of the C-terminal label changes.
5   Any decrease in signal intensity is an indication of cleavage
inhibition. Other permutations and possibilities will be
readily apparent to those of ordinary skill in the art, and
are considered as equivalents within the scope of the instant
invention.

10   C.   Examples

The examples presented below are intended to be
illustrative of the various methods and compositions of the
invention.

EXAMPLES

15   Example 1:   Purification Scheme

A.   Protein Purification from Brain Tissue

Frozen human brain tissue (500 g wet weight) was thawed,
then homogenized in a Waring blender with three parts (v/w) of
ice-cold 20 mM Tris, pH 7.5, 2 mM EDTA, 5 mM 2-mercaptoethanol
20   ("Buffer A"). The mixture was centrifuged at 10,000 x g for
60 min at 4°C, and the pellet discarded The supernatant was
recentrifuged at 15,000 x g for 60 min.

About 1 1 of the centrifuged solution was applied to a
100 ml packed DE-52 diethylaminoethyl anion exchange column
25   pre-equilibrated with Buffer A. After loading for 8 hr at
4°C, the column was washed with 10 volumes of 1 1 of Buffer A,
then eluted with 60 mM NaCl in Buffer A. Fractions of the
eluate containing peptide cleavage activities were pooled and
concentrated in an Amicon pressure cell to 5 ml.
30   The concentrated eluate was chromatographed on a 2.5 x
100 cm S-200 Sephacryl (Pharmacia) molecular sieving column,
equilibrated with Buffer A supplemented with 100 mM NaCl, and
chromatographed ov rnight at 4°C at 1 ml/min. All three
peptide cleavage activities eluted in a symmetrical peak with
35   an apparent MW of 80,000. Th fractions containing peptide

cleaving activity were pooled, then dialyzed for 16 hr at 4°C
into 10 mM sodium phosphate, pH 7.5, containing 10 uM Ca⁺⁺.

Th dialyzat was then loaded on to a Bio-Gel HT column
(Bio-Rad) (40 ml bed volume) pre-equilibrated with the same

5   buffer used for dialysis at 1 ml/min at 4°C, washed with five
volumes of the buffer, and then eluted with a 250 ml 10 to 250
mM sodium phosphate linear gradient. Tne protease activity
eluted at approximately 100 mM sodium phosphate. The sharp
peak of activity was pooled, made 1 M in ammonium sulfate (by

10   dilution with 3 M ammonium sulfate in 100 mM sodium phosphate,
pH 7.5) centrifuged at 15,000 x g at 4°C for 20 min, and the
supernatant loaded at 4°C ontc a 7.5 x 75 mm HPLC Phenyl-TSK
column equilibrated in 1 M ammonium sulfate in 100 mM sodium
phosphate, pH 7.5. The column was washed with 10 ml of the

15   equilibration buffer, and eluted using a linear gradient in
which the ammonium sulfate concentration decreased from 1 M to
0 M. The amyloidin fractions eluted at approximately 0.4 M
ammonium sulfate and were pooled.

The purification steps described here are important,

20   since at least one major contaminating protease activity is
separated out at the Phenyl-TSK step. This activity, which is
not affected by Ca⁺⁺, and cleaves the peptide at the Ala-Glu
bond, elutes early from the column, followed by the
characteristic three-site cleavage pattern of amyloidin. The

25   chromatogram shown in FIG. 1 and developed from the material
eluted from the Phenyl-TSK HPLC column, shows the
characteristic three-site cleavage by amyloidin. Although we
were able to detect an activity which we designated "amyloidin
II" that also cleaved the substrate at the Met-Asp bond, this

30   activity could not be reproducibly isolated.

The pooled material was dialyzed at 4°C for 4 hr against
25 mM bis-Tris, pH 6.3 and then applied to a 1.5 x 30 cm PBE
94 Chromatofocusing® column (Pharmacia) pre-equilibrated with
the 25 mM bis-Tris, pH 6.3 buffer. The column was eluted with

35   a decreasing pH gradient from 6.3 to 3.0 using Polybuffer 74
diluted 1/8 with water, and adjusted to pH 3.8 with HCl.
Amyloidin fractions eluted at an approximate pH of 4.3.
SDS-PAGE (reducing conditions) analysis of fractions

containing amyloidin activity showed a predominant band at
80,000, as estimated by the relative mobility against low
molecular weight protein markers purchased from Bio-Rad
(Richmond, CA).

5   B.   Protein Purification of Amyloidin from Blood
         Outdated blood was obtained from the blood bank. Six
units of whole blood were centrifuged at 2,000 x g for 30 min,
and the plasma and buffy coat discarded. The packed cells
were washed four times with Buffer A plus 140 mM NaCl, with
10   centrifugation at 2,000 x g for 30 min and discarding the wash
after each step. The collected, washed erythrocytes were
lysed by osmotic shock in 6 volumes of 5 mM Tris-HCl, pH 7.5,
2 mM EDTA and 5 mM 2-mercaptoethanol, for 30 min on ice, then
centrifuged at 15,000 x g for 60 min at 4°C.
15   The supernatant was mixed with DE-52 (Whatman)
pre-equilibrated with Buffer A using approximately 85 ml of
settled bed volume for the ion exchanger per unit of whole
blood. The DE-52 slurry was stirred for 1 h on ice, then
washed in a Buchner funnel with 8 L of the Buffer A solution.
20   The washed ion exchanger was packed into a glass column,
washed with 4 L of Buffer A, then eluted with 60 mM NaCl in
Buffer A.
         The pool of peptide-cleavage activity was dialyzed
against 4 L of 10 mM sodium phosphate, pH 7.5, 10 μM CaCl$_2$,
25   and the dialyzate passed through an Affigel Blue column (50 ml
bed volume, Bio-Rad). All the peptide cleavage activity
passed through the column unretarded. The solution was
collected and loaded onto a 100 ml Bio-Gel HT column
pre-equilibrated with the dialyzing buffer. The column was
30   then washed with 100 ml of the same buffer and eluted with a
linear gradient of 10-250 mM sodium phosphate, pH 7.5, 10 μM
CaCl$_2$, total volume 600 ml. The peak of peptide cleavage
activity was pooled, then dialyzed against 25 mM Bis-Tris, pH
6.3, and applied to a 1.5 x 40 cm PBE 94 (Pharmacia)
35   Chromatofocusing® column.
         The Chromatofocusing column was washed with 200 ml of
the loading buffer and the retentant eluted with a decreasing

pH gradient from 6.3 to 3.8, using Polybuffer 74 diluted 1:8
with water and then the pH was adjusted to 3.8 with HCl. The
amyloidin activity (eluting at approximately pH 4.3) was
pooled, and then dialyzed against 100 mM sodium phosphate, pH
5   7.5, 1 M ammonium sulfate. The dialyzate was then loaded onto
a 7.5 mm x 75 mm HPLC Phenyl-TSK column (Toyo-Soda, Japan)
equilibrated with the dialysis buffer, washed with 10 ml of
the equilibration buffer, then eluted using a linear gradient
in which the ammonium sulfate concentration decreased from 1 M
10   to 0 M. The amyloidin fractions eluted at approximately 0.4 M
ammonium sulfate and were pooled.

        C.   Alternative Protein Purification Scheme
        Following initial tissue extration procedures as
described for brain tissue (1A) or blood (1B) above, the
15   soluble extract was mixed with DE-52 (Whatman) pre-
equilibrated with 20 mM Tris, pH 7.5, 2 mM EDTA, 5 mM
β-mercaptoethanol, using approximately 100 ml packed resin per
liter of extract.
        Peptide cleavage activity was pooled and dialyzed against
20   4 l of 10 mM sodium pohosphate, pH 7.5, 10 μM CaCl₂, then
applied to a 100 ml Bio-Gel HT column pre-equilibrated with
the dialyzing buffer. The elution of this column and
subsequent steps, e.g., PBE 94 (Pharmacia) Chromatofocusing®
and HPLC phenyl-TSK chromatography, were done exactly as
25   described in Example 1B above. The exact order of these
subsequent steps may be performed in a different sequence than
that presentedin this example. When phenyl-TSK chromatography
preceded PBE 94 Chromatofocusing®, similar purification and
yields were obtained.

Fraction	Volume (ml)	[Protein] (mg/ml)	Total Protein (mg)	Total Activity DAEFR area, mV-sec/min)	% Yield	Specific Activity (area/min/mg protein)	Fold Purification
Crude	4200	236	991200	114371	100.00%	0.12	1
DE52	165	10.1	1666.5	422334	369.27%	253.43	2196
HT	142	1.4	198.8	59252	51.81%	298.05	2583
PBE 94	150	0.54	81	62125	54.32%	766.96	6647
Phe TSK	40	0.14	5.6	11644	10.18%	2079.29	18020

30   D.   Enzymatic Assay
        The peptides of the invention can be prepared by solid
phase synthesis (Kent and Lewis in "Synthetic Peptides in

Biology and Medicine," Alitalo, ed. (1985) Elsevier) or by
other standard peptide synthetic means. APP592-601 was
synth sized by Applied Biosystems (Foster City, CA) and the
anhydrous hydrogen fluoride (HF)-crude further purified by
5   reverse-phase HPLC. The composition was confirmed by amino
acid analysis on an Applied Biosystems 420 Automated Amino
Acid Analyzer. APP592-601 has a free carboxy terminus which
is preferred for purposes of the cleavage assay described
below, whereas the analogs synthesized below have a C-terminal
10   amide.

        Routinely, 25 μl of enzyme solution were mixed with 10 μl
of 1 M Tris, pH 7.5, 5 μl 10 mM CaCl$_2$, and 10 μl of a 2 mg/ml
stock solution of APP592-601 in water, or 20 mM Tris, pH 7.5,
0.15 M NaCl, in 1.5 ml polypropylene microfuge tubes. The
15   reaction mixtures were incubated for 60 min at 37°C in a water
bath, then quenched with 450 μl ice-cold ethanol, and
incubated on ice for a further 20 min. They were centrifuged
at 15,000 x g for 10 min, and the supernatants transferred to
new polypropylene tubes and dried under vacuum. The residue
20   was dissolved in 0.5 ml water, centrifuged at 15,000 x g for 5
min, and 200 μl of the supernatant injected onto a 0.46 x 30
cm Vydac C18 column equilibrated with 0.1% trifluoroacetic
acid in water, at 1 ml/min. The column was immediately eluted
with a linear gradient to 40% acetronitrile in 0.1%
25   trifluoroacetic acid in water, over 20 minutes. The elution
was monitored at 220 nm, and peaks were individually
collected, hydrolyzed in 6 N HCl at 65°C for 2 hr, then
subjected to quantitative amino-acid analysis in an Applied
Biosystems amino-acid analyzer. Once identified, times of
30   elution were used to identify cleavage patterns. The
characteristic three-site cleavage pattern of amyloidin is
shown in FIG. 1.

        Example 2: Enzymatic Properties
                Amyloidin is strongly inhibited by EDTA, since
35   substitution of 5 μl 100 mM EDTA for the CaCl$_2$ in the standard
peptide cleavage assay described in Exampl 1D, leads to no
detectable cleavage of the APP592-601 by this protein.

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The molecular weight estimate for the purified amyloidin was made by comparing the mobility of the Coomassie-stained band with that of low molecular weight standard protein markers (FIG. 2, Lanes 1 and 4) supplied by Bio-Rad

5   (phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 42,700; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400); Lane 2 is amyloidin (non-reducing); and Lane 3 is amyloidin (reducing).

The inhibition of amyloidin by various inhibitors was

10   tested by individually pre-incubating the enzyme with the inhibitor compounds listed in the following table for 30 min at room temperature, prior to addition of APP592-601 to start the reaction. The conditions for each reaction are also provided in the table. The solvent stock solution of

15   inhibitor is made up in water unless otherwise indicated (DMSO, dimethylsufoxide; EtOH, ethanol). Percent of activity of amyloidin is shown, as calculated with respect to control without inhibitor but with appropriate solvent.

Inhibition of Amyloidin

Inhibitor*	Stock conc.	Assay conc.	% Control Activity
PMSF	50 mM EtOH	1 mM	69
DFP	20 mM EtOH	2.5 mM	0
EDTA	100 mM	10 mM	0
E-64	10 μg/ml	1 μg/ml	119
1,10-phen.	5 mM DMSO	0.1 mM	75
phosphoramidon	1 mg/ml	0.1 mg/ml	79
Calp. Inh. II	2.5 mg/ml	50 μg/ml	130
chymostatin	1 mg/ml DMSO	25 μg/ml	77
aprotinin	0.1 mg/ml	2 μg/ml	102
α1-PI	1 mg/ml	30 μg/ml	98
α1-ACT	1 mg/ml	30 μg/ml	121

* PMSF, phenylmethylsulfonyl fluoride; DFP, Diisopropyl-fluorophosphate; EDTA, ethylenediaminetetraacetic acid; 1,10-phen., 1,10-phenanthroline; calp. inh. II, calpain inhibitor II; α1-PI, α1-proteinase inhibitor; α1-ACT, α1-antichymotrypsin.

Calpain inhibitor-II or E-64 (both strong inhibitors of the Ca^{++}-dependent cysteine protease, calpain), had no inhibitory effect on amyloidin. 1,10-phenanthroline and phosphoramidon, both strong inhibitors of metalloproteases, were only w akly inhibitory (25% inhibition). Neither

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α-1-proteinase inhibitor or α-1-antichymotrypsin, two general plasma serine proteinase inhibitors, had any inhibitory effect on amyloidin, nor did aprotinin, the bovine Kunitz trypsin inhibitor.

5   Example 3:   <u>Structural Characterization</u>

   A.   Amyloidin

   To obtain sequence information, approximately 300 pico-moles of amyloidin purified from human brain tissue (Example 1) were electrophoresed on a 7.5% acrylamide gel using

10   SDS-PAGE, and the protein band was visualized with Poinceau Red (stock solution from Sigma diluted 1:10 with water).

   The band was excised from the gel and minced into small pieces with a clean razor blade. 30 picomoles of Lys-C endopeptidase (Boehringer Mannheim) in 100 µ 50 mM Tris-HCl,

15   pH 8.5, 1 mM EDTA, was then added to the protein, and incubated overnight. The gel pieces were repeatedly extracted with 10 mM ammonium bicarbonate in acetonitrile (5 x 100 µ), the washings combined and dried. The dried gel samples were then taken up in 50 µ 0.1% TFA, and injected into a 0.2 cm x

20   15 cm Vydac C18 micropore column, and eluted with a linear gradient of 0-60% acetonitrile over 60 min. The three peaks that were deemed most pure were then sequenced to completion or to whatever was practical on an Applied Biosystems 470A automated protein sequencer, with online PTH analysis, using

25   the programs supplied by the manufacturer.

   The three most prominent peaks were sequenced and are provided below:

   H_2N-Arg-Val-Tyr-Asp-Gln-Val-Gly-Thr-Gln-Glu-Phe-Glu-Asp-Val-Ser-Tyr-Glu-Ser-Thr-Leu-Lys-COOH (Seq. ID No.2);

30   H_2N-Val-Asp-Gln-Ala-Leu-His-Thr-Gln-Thr-Asp-Ala-Asp-Pro-Ala-Glu-Glu-Tyr-Ala-Arg-Leu-Cys-Gln-Glu-Ile-Leu-Gly-Val-Pro-Ala-Thr (Seq. ID No:3); and

   H_2N-Glu-Tyr-Phe-Pro-Val-Gln-Val-Val-Thr-His-Gly-Leu-Leu-Gly-Ile-Tyr-Gln-Glu-L u-Leu-Gly-Leu-Ala-Ph -His-His

35   (S q. ID No:4).

Direct attempts at s quencing the 50,000 MW band, by transferring to Immobilon membranes (M.llipore) were unsuccessful, which indicated a probable blocked N-terminus.

Example 4:   Substrate Specificity

5   The ability of amyloidin to cleave short peptide-based para-nitroanilide substrates was tested by incubating 10 μ of a 20 mM stock of peptide p-NA substrate with 25 μ of enzyme, 20 μ of 100 mM CaCl,, 40 μ of 1 M Tris-HCl, pH 7.5, and 105 μ water, in 96-well microtiter plates, and monitoring for

10   increase in absorbance at 405 nm, in a Molecular Devices Vmax Kinetic Microplate Reader. The results are provided below. No measurable increase in A_{405} was detected, even after incubations up to 2 hours. Thus, amyloidin does not appear to cleave the pNA substrates tested, including one which is

15   derived from the APP592-601 sequence.

Substrate*	Amyloidin
Suc-AAPM-pNA	0
MeS-AAPV-pNA	0
Suc-AAPF-pNA	0
Ac-AD-pNA	0
Boc-AAd-Pna	0
Z-AA-pNA	0
Z-RR-pNA	0
Z-RK-pNA	0
Ac-EVKM-PNA	0

* Sub-AAPM-, Sucinyl-alanyl-prolyl-methionyl-; MeS-AAPV-, Methoxysuccinyl-alanyl-alanyl-prolyl-valyl-; Suc-AAPF-, Succinyl-alanyl-alanyl-prolyl-phenylalanyl-; Ac-AD-, Acetyl-alanyl-aspartyl-; Boc-AAD-, Butyloxycarbonyl-alanyl-
30   alanyl-aspartyl-; Z-AA-, benzyloxycarbonyl-alanyl-alanyl-; Z-RR-, benzyloxycarbonyl- arginyl-arginyl-; Z-RK-, benzyloxycarbonyl-arginyl-lysyl-; Ac-EVKM-, acetyl-glutamyl-valyl-lysyl-methinyl-pNA; pNA-, para-nitroaniline

Amyloidin does not appear to cleave oligopeptide
35   para-nitroanilide substrates, including one based on the APP sequence after which it cleaves in the APP592-601 peptide.

Various peptides were purchased from Bachem (Torrance, CA) and incubated with the human amyloidin protease using the conditions described in Exampl 1D. Cleavage products were
40   isolated by reverse-phase HPLC, and subjected to amino acid analysis to determine sit (s) of cleavage. The following table show the results obtained with amyloidin protease compared to report d site(s) of cl avage by Pz-p ptidase.

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Substrate	Cleavage Products
Bradykinin	ArgProProGlyPhe SerProPheArg
Neurotensin	GluLeuTyrGluAspLysProArg ArgProTyrIleLeu
LH-RH	GluHis TrpSerTyr GlyLeuArgProGlyNH₂
	_____ (minor)
Dynorphin A108	TyrGlyGlyPheLeu ArgArgIle
	_____(minor)

The spaces indicate cleavage sites of Pz-peptidase reported by
Barrett (1990), supra. Underlined peptides are those
identified following cleavage by the amyloidin of the present
invention.

Example 5: **Subsite Requirements**

        In order to test the subsite requirements of amyloidin in
the APP592-601 cleavage assay, analogs of this peptide
substrate were synthesized.

        A.   **Chemical Synthesis of (N-acetyl)-APP (592-601)**
              CONH₂ analogs

        The peptide corresponding to residues 592 to 601 of the
695 APP was synthesized on the Applied Biosystems Model 430A
Peptide Synthesizer using the t-boc methodology. All
boc-amino acids and synthesis reagents were purchased from
Applied Biosystems Inc., and the amino acid side chain
protecting groups are as follows: Arg(TOS), Asp(OBzl),
Lys(2ClZ), and Ser(OBzl). Ala, Met, Phe, Gln, Nleu and Val
were used with no side chain protecting group. [(TOS) -
tosyl, (OBzl) - O benzyl, (2ClZ) - 2,6-dichlorocarbobenzoxy].
The software for controlling the synthetic cycle was designed
specifically for making long chain peptides and peptides with
sequence specific coupling difficulties. The general cycle is
as follows: boc deprotection, neutralization; amino acid
activation, 1st coupling (in CH₂Cl₂), neutralization; amino
acid activation; 2nd coupling (in dimethylformamide),
neutralization; and finally acetic anhydride capping.
        Boc-amino acids were activated as preformed symmetric
anhydrides by addition of 0.5 equivalents of
N,N-dicyclohexylcarbodiimide (DCC) with the exception of
boc-Arg(TOS) which was activat d to its corresponding

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HOBT-ester by addition of 1 equivalent of
1-hydroxybenzotriazole and 1 equival nt of DCC. After the
s cond coupling of each amino acid, any uncoupled amine
remaining was capped by addition of acetic anhydride in CH₂Cl₂
5   plus a catalytic amount of diisopropylethylamine (DIEA). The
capping step is to prevent synthesis of deleted peptide
sequences which are often difficult to separate from the
target peptide during purification.

    After addition of the N-terminal amino acid, the boc
10 group was removed using 50% (TFA) in CH₂Cl₂ and neutralized
with a solution of 10% DIEA in CH₂Cl₂. The exposed primary
amine of the N-terminal amino acid was then acetylated using
the same protocol using the capping step.

    The solid support, p-methylbenzhydrilamine resin, was
15 purchased from Fisher Biotech. Treatment of the fully
protected peptide resin with anhydrous HF, cleaved the peptide
from the solid support, removed all the side chain protecting
groups, and produced the crude peptide product as the
C-terminal carboxy amide derivative.

20   The crude peptide was purified to >98.0% purity using
preparative scale reverse phase chromatography on a Vydac,
C18, 330A, 10um column with dimensions of 2.2 cm x 25 cm in
length. The crude peptide was dissolvea and loaded onto the
column in 5%, [0.1% TFA/CH₃CN]/H₂O and eluted using a linear
25 gradient of 5% to 50% [B] over 135 minutes. ([B] = 0.1%
TFA/CH₃CN).

    The structural integrity of the purified peptide was
assessed by analytical HPLC, amino acid composition analysis,
and mass spectrometry.

30   Following synthesis and purification, the peptides were
then incubated with amyloidin under conditions identical to
those developed with APP592-601. The results are summarized
below. The underlined residues designated changes from the
native peptide sequence while the arrows indicate peptide
35 cleavage sites.

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                        ↓   ↓   ↓
        Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg
                              ↓
        Ser-Glu-Val-Gln-Met-Asp-Ala-Glu-Phe-Arg
5   ↓   ↓
        Ser-Glu-Val-Lys-Ala-Asp-Ala-Glu-Phe-Arg
                        ↓   ↓   ↓
        Ser-Glu-Val-Arg-Met-Asp-Ala-Glu-Phe-Lys
                        ↓   ↓   ↓   ↓
10   Ser-Glu-Val-Lys-Nle-Asp-Ala-Glu-Phe-Arg

        With the exception of the analog in which Lys595 was
replaced with a Gln, amyloidin cleaved at multiple sites,
which differed from peptide to peptide. The replacement of
Met596 by Ala resulted in loss of cleavage at this site, but
15 cleavage shifted to the Lys595-Ala596 bond. In the Gln595
analog, all the cleavage by amyloidin was at the Met-Asp bond.
        While amyloidin has been demonstrated to make the
relevant cleavage at the Met-Asp bond of synthetic amyloidin
substrates, under certain preliminary conditions tested, the
20 protease did not cleave the full-length protein (APP) produced
by baculovirus cells transformed with the gene encoding APP.
However, this does not preclude the involvement of this enzyme
in the processing of APP as amyloidin may be only one of
several enzymes whose combined activity is necessary for the
25 generation of the β-peptide from APP.

Example 6:   Purification of Clipsin/RMCP I
        The procedure to isolate RMCP I was adapted exactly from
that described by Nelson & Siman (1990), supra.
However, after the final extraction of the membrane pellets
30 with 50 mM HEPES, pH 7.5, 1 M MgCl,, and 0.1% Brij 35, the
soluble extract was not further dialyzed, but loaded onto an
immobilized soybean trypsin inhibitor column. This protocol
and the subsequent steps were adapted from that taught from
the purification of RMCP I described in Woodburg, et al,
35 supra.

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The soybean trypsin inhibitor column was prepared as
follows: 15 mg of soybean trypsin inhibitor (Sigma Chemicals)
was dissolv d in 10 ml of 0.1 M sodium bicarbonate, pH 8.5,
and mixed with 3 g of washed activated CH-Sepharose (Sigma)
5   for 2 h end-over-end in a plastic tube. The gel slurry was
then filtered over a coarse sintered glass funnel, and
extensively washed with alternating buffers, 0.1 M formate, pH
3, and 0.2 M Tris-HCl, pH 8. The washed gel was re-suspended
in 0.2 M Tris, pH 8, then equilibrated with 50 volumes of the
10   same buffer after loading in a small glass column. The gel
was then equilibrated with 50 mM HEPES, pH 7.5, 1 M MgCl,, and
0.1% Brij 35. The RMCP I extract was then loaded onto the
column, and the flow-through material re-applied three times.
(Measurement of Suc-Ala-Ala-Pro-Phe-pNA hydrolysis, which was
15   used by Nelson & Siman to assay for "clipsin" activity,
indicated that 95% of this activity had bound to the matrix.)
The column was then washed with 5 volumes of the loading
buffer, then eluted with 10 ml of 25 mM formate, pH 3, 0.1
mg/ml bovine serum albumin. The eluate was collected into an
20   equal volume of 0.2 M ammonium bicarbonate, pH 8.6, with 0.1
mg/ml BSA. This pool was diluted 1:1 with distilled water and
treated with 1 g of reagent grade barium sulfate (Aldrich) for
30 min on ice. The supernatant was decanted and the pellet
washed with 5 ml of 10 mM Tris, pH 8. The pellet was
25   extracted with 2 ml of 20 mM Tris, pH 8 and 1 M NaCl. This
extract was assayed for both Suc-Ala-Ala-Pro-Phe-pNA
hydrolysing activity, as well as APP592-601 cleaving activity.
APP592-601 was cleaved at the Met-Asp bond, and this protease
activity was completely inhibited by pre-treating the protease
30   aliquot with 200 ng of α_1-antichymotrypsin, which had been
shown to be a strong inhibitor of RMCP I. This pre-treatment
also completely eliminated the measured
Suc-Ala-Ala-Pro-Phe-pNA hydrolysing activity.
        Incubation of 200 ng each of RMCP I and human skin
35   chymase (HSC), obtained from Dr. Norman Schecter, University
of Pennsylvania, with APP592-601 in the standard peptide
cleavage assay also resulted in cleavage at the Met-Asp bond.
Since the latter two enzym s are known to hydrolyze

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Suc-Ala-Ala-Pro-Phe-pNA, and are also inhibitable by
α₁-antichymotrypsin, this suggested that RMCP I and HSC all
belong to a s cond class of proteases, distinct from
amyloidin, but also able to cleave the APP592-601 at the
5   Met-Asp peptide bond.

Example 7:   Production of Antibodies
        For the generation of antibodies to amyloidin, two
approaches were taken. A peptide sequence corresponding to
sequence 1 obtained from structural analysis of amyloidin, but
10   having an additional Cys at the amino-terminus was synthesized
by the standard synthesis methods described earlier. About 72
mg of the crude HF-cleaved peptide
(Cys-Val-Tyr-Asp-Gln-Val-Gly-Thr-Gln-Glu-Phe-Glu-Asp-Val-Ser-
Tyr-Glu-Ser-Thr-Leu-Lys) were conjugated to 12 mg of rabbit
15   serum albumin (RSA), using Sulfo-MBS (Pierce, USA) in 50 mM
potassium phosphate, pH 7 (Lerner, et al (1981) Proc Natl Acad
Sci USA 78:3403-3407). The conjugation reaction was allowed
to proceed for 20 min on ice, then immediately separated on a
Sephadex G-10 column. The conjugated protein pool was sent to
20   Josmin Laboratories (Berkeley, CA) for injection into rabbits.
Briefly, primary immunization was done with 0.5 mg of
conjugated RSA, followed by a first boost after three weeks.
The first bleed obtained two weeks after the last of three
weekly injections was tested by Western blot analysis for
25   reaction against purified amyloidin. Strong reaction was
obtained in the anti-serum from one of the two rabbits
injected, and this serum was further processed by isolating a
crude IgG fraction by sodium sulfate fractionation. As shown
in FIG. 3A and 3B, a Western blot using the IgG fraction
30   diluted 1:500 as the primary antibody, the 80,000 dalton
protein band in amyloidin is exclusively recognized. Lane 1
(FIG. 3A) shows pre-stained Bio-Rad low molecular weight
markers and Lane 2 (FIG. 3B) shows Amersham Rainbow markers;
Lane 2 (FIG. 3A) and Lane 1 (FIG. 3B) show th purified
35   amyloidin.
        In the second approach, 60 µg of purified amyloidin were
electrophoresed on SDS-PAGE, and the 80,000 dalton amyloidin

band visualized by Rapid Reversible Stain (Diversified
Biotech). The protein band was excised with a razor blade,
then destained according to the manufacturer's instructions.
The gel strip was then cut into small pieces and homogenized
5   with a small amount of phosphate buffered saline (PBS) and
emulsified into a water-in-oil emulsion with Freund's complete
adjuvant by repeated passage through an emulsifying needle.
Aliquots (20 μg) of this were injected into three Balb/C mice.
This procedure was repeated every other week, with the
10   modification that subsequent emulsifications were done with
Freund's incomplete adjuvant. The serum of each mouse was
tested for reactivity against purified amyloidin by Western
blot analysis after three injections. The mouse whose serum
showed the strongest reactivity was further selected for
15   fusion with myeloma cells to generate hybridomas producing
antibodies against amyloidin. The hybridomas were generated
by standard murine fusion procedures as described in
ANTIBODIES: A Laboratory Manual by Harlow & Lane (Cold Spring
Harbor Laboratories, 1988). Briefly, the immunized mouse was
20   sacrificed and the spleen removed. Mixed splenocytes were
obtained by pressing the spleen between frosted ends of glass
slides. These were fused with SP2/0Ag14 plasmacytoma cells
(ATCC No. CRL1581) at a fusion ratio of 1:3 in Dulbecco's
modified Eagle's media (DMEM), supplemented with 20% fetal
25   bovine serum (FBS), 2 mM glutamine, 15 mM HEPES and 0.1 mM
hypoxanthine. Hybridomas were selected for by growing the
cells in the presence of azaserine supplemented DMEM,
augmented with hypoxanthine and 20% FBS. Hybridomas were
screened for reactivity against purified human brain amyloidin
30   using an ELISA. Positives were further tested in Western
blots and the results are shown in FIG. 4. From this single
source, 10 monoclonals have been generated which recognize
purified amyloidin strongly on Western blots.

Example 8:   Immunohistological Studies
35   Brain tissue from Alzheimer disease (AD) patients and age
matched controls was immersion fixed in 4.0% paraformaldehyde
in 0.1M phosphate buffer and cut into 40 μm sections on a

sliding microtome. Sections were collected in 0.1M phosphate
buffer and quenched for endogenous peroxidase activity for 20
minutes in 0.3% hydrogen peroxide and 0.5% Triton X-100 in
0.1M phosphate buffer. They were blocked for 1.0 hour in 5.0%
5   milk in phosphate buffer and then incubated for 24 hours in
the polyclonal antibody to amyloidin protease diluted 1:20 in
1.5% goat serum and phosphate buffer. As controls, some
sections were incubated at the same concentration with
preimmune sera from the rabbit producing the antibody and
10 antibody adsorbed with the amyloidin peptide. They were then
processed for immunocytochemistry using standard procedures
for the goat anti rabbit IgG Vectastain ABC kit (Vector
Laboratories). Brain tissue from rats transcardially perfused
with 9.9% NaCl followed by PLP fixative was similarly
15 processed. In addition, similar tissue from rat, AD and age
matched control brains was embedded in paraffin and cut into 8
μm sections. These sections were baked for 1-3 hours and
hydrated by passing the sections for 3 minutes each in three
changes of xylene, two changes of 100% ETOH, and one change
20 each through 95%, 70%, 50% EtOH and dH,O). These were also
routinely processed with the Vectastain ABC kit. To determine
the efficacy of formic acid pretreatment, some of the paraffin
embedded material was incubated with formic acid for 3 minutes
following hydration.
25   In both normal and AD brains the antibody stained large
neurons in layers III and IV, white matter astrocytes, and
smooth muscle cells. In AD brains large reactive astrocytes
were stained in the gray matter. This staining was not seen
with preimmune sera and was blocked by adsorbing the antibody
30 with the peptide antigen. In rat brain, a number of
astrocytes in the hippocampus were labeled; no neurons were
labeled.
        In paraffin embedded AD sections without formic acid
pretreatment only smooth muscle cells and a few, presumably
35 reactive, astrocytes in the gray matter w re labeled; no
obvious pathology was stain d. However after formic acid
pretreatment, the predominant staining in the same brains was
associated with AD pathology; dystrophic neurites, notably

those forming neuritic plaques were labeled. Several large
n urons were also labeled, however they were not found evenly
distributed throughout the gray matter. Instead they were
found in clusters, usually in layer V. Smooth muscle cells
5   were also labeled.

In rat paraffin embedded material, no cortical neurons
were labeled before or after pretreatment with formic acid.
Only smooth muscle cells were seen in cortical arterioles,
little or no labeling was seen in the cerebellum. A few
10   neurons were labeled in the midbrain.

In summary, the staining patterns of the polyclonal
antibody against amyloidin protease indicate that it may be
abundant in human brain cells and may be highly expressed in
the cellular components underlying the pathology associated
15   with Alzheimer's Disease. The reason why formic acid
pretreatment changes the staining pattern in paraffin embedded
in tissue is unclear; the compromised epitope may be altered
or obscured in dystrophic versus normal cells and neurites.
The lack of neuronal staining in the rat is interesting and
20   may signify a difference in degradation products between the
two species.

Example 9:   Cloning of the Gene Encoding Human Amyloidin
Total RNA was extracted from normal human brain (patient
ID 87-5); superior temporal gyrus and a human embryonic kidney
25   cell line; 293 (ATCC No. CRL1573). As shown in Example 1,
human brain homogenates have amyloidin activity. Similarly,
293 cell extracts were shown to have amyloidin activity and
therefore cDNA made from this RNA would be a preferred
template for amyloidin sequence identification.
30   Complementary DNA was generated using random hexamers.
PCR primers were designed to give the longest PCR product of
region III (Seq. ID No:4). The sequence of the PCR primers
(EJ-87 and EJ-88) were based on Lathe's rules ((1985) J Mol
Biol 183:1-12) although degenerate primers did work also (EJ-
35   91, EJ-92 and EJ-93).
     EJ-87: 5' TCGAATTC AAG GAG TAC TTC CCT GT 3' (Seq ID No:7
     EJ-88: 5' CAAAGCTT TG GAA GGC CAG GCC CAG 3' (Seq ID No:8)

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EJ-89: 5' CAT GGC CTG CTG GGC ATC TAC CAG GAG 3' (Seq ID No:9)

EJ-91: 5' TCGAATTC AAR GAR TAY TTY CCN GT 3' (Seq ID No:10)

EJ-92: 5' CAAAGCTT RTG RTT NGC NAG NCC 3' (Seq ID No:11)

EJ-93: 5' CAAAGCTT RTG RTT NGC YAA NCC 3' (Seq ID No:12)

5   where N can be A, T, C or G; R can be A or G; and Y can be C
or T.

PCR products were analyzed by Southern blot hybridization
using internal probe EJ-89. PCR reactions were performed at
95°C; 1 min denaturing, 42°C, 48°C or 54°C; 1 min annealing;
10 72°C, 1 min extension times for 35 cycles. A 93 bp product of
the EJ-87/88 reaction that did hybridize with EJ-89 was
excised from an analytical acrylamide gel and used as template
for a re-PCR reaction. DNA sequencing was performed using EJ-
87 and EJ-88 as primers as described by Smith, et al (1990)
15 Biotechniques 9:51. A unique DNA sequence of approximately 40
base pairs was then provided for further cloning efforts.

This 40 base pairs of unique sequence, along with
flanking sequence from the PCR oligos EJ-87 (Seq ID No:7) and
EJ-88 (Seq ID No:8), were used to generate an oligo probe,
20 designated 811 (Seq ID No:13) 5' AAGGAGTACT TCCCTGTGCA
GGTGGTCACG CACGGGCTGC TGGGCATCTA CCAGGAGCTC CTGGGCCTGG
CCTTC 3' to screen libraries for amyloidin clones.

Because this probe contains at least 40 contiguous base
pairs of exact sequence, stringent conditions (55°C, 0.1X SSC
25 wash) were used during screening; otherwise screening
techniques were standard (see Sambrook, et al (1990)). This
probe was used to screen a temporal cortex cDNA library
(obtained from Stratagene, catalogue number 935205), and one
clone (clone 19) was obtained. Partial sequence generated
30 from clone 19 was used to design the following PCR oligos:

895 (Seq ID No:14) 5' GAAATGCACG TGCCTGAG 3'

889 (Seq ID No:15) 5' CCAGGACATA GTCGGCG 3' (antisense)

that were used to generate a double-stranded PCR probe from
the 5' end of clone 19. Although the Stratagene library was
35 screened with this probe (Seq ID No:16) 5' GAAATGCACG
TGCCTGAGAC CAGGAGGAAA GTGGAGGAGG CCTTCAACTG CCGGTGCAAG
GAGGAGAACT GCGCTATCCT CAAGGAGCTG GTGACGCTGC GGGCCCAGAA
GTCCCGCCTG CTGGGGTTCC ACACGCACGC CGACTATGTC CTGG 3', no clones

containing the 5' end of the coding region of amyloidin were
obtained.

        Western analysis showed that HeLa cells also contain
amyloidin. A standard HeLa cell random primed cDNA library in
5   the lambda gt10 vector was provided by Dr. Bernhard Luscher
(University of California, Berkeley). Commercially available
HeLa cell cDNA libraries are available from Stratagene
(catalogue number 936201) and Clontech (catalogue number
HL1022b). This library was screened with the same PCR probe
10   generated from oligos 889 and 895 described above, and 30
positive clones were plaque purified. The amounts of 5' and
3' flanking sequences in all of the positive clones were
estimated by PCR analysis using oligos homologous to the
lambda vector sequences (from Clontech, catalogue number 5411-
15   1) and either an oligo made to the 5' end of clone 19
(antisense strand; oligo 909; 5' ACTTTCCTCCTGGTCTCA 3') (Seq
ID No:17) or to the 3' end of clone 19 (coding strand; oligo
905; 5' GGAGAAGCTCATTGAGTC 3') (Seq ID No:18). Sizing of the
PCR products was done by agarose electrophoresis, and those
20   clones with the most flanking sequences were chosen for
sequence analysis. These clones were cut out of the lambda
vector and subcloned into M13 for sequencing. Two clones,
clones cHL57 and cHL53, which together span the entire coding
region of amyloidin, were chosen for complete sequence
25   analysis.

        The coding region of human amyloidin is provided as Seq
ID No:6. The complete sequence of the human amyloidin gene
was obtained by sequencing two clones: clone cHL57 provided
the nucleotide sequence encoding amino acid residues 1 through
30   480, with Met1 of Seq ID No:5 being the putative initiation
codon; clone cHL53 provided the nucleotide sequence encoding
amino acid residues 56 thorough the stop condon of the
amyloidin protease. Examination of the nucleotide sequence
reveals that there is no clear hydrophobic leader sequence as
35   would be expected for a secreted or membrane-bound protein.
This is similar to that found by Pierotti, et al (1990), supra
for the rat Pz-peptidase. Howev r, the human amyloidin
sequence, in contrast to the rat Pz-peptidase sequence,

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contains approximately 132 base pairs of additional coding
DNA, and therefore codes for an enzyme having approximately 44
additional amino acid residues at the carboxy-terminus.

The open reading frame of the cDNA encoding human

5   amyloidin is composed of 2070 nucleotides, including the stop
codon (Seq ID No:6), encoding a protein with 689 amino acids
residues (Seq ID No:5). Like other members of the family of
zinc-dependent metallopeptidases, human amyloidin contains the
typical amino acid sequence at and around the active site that

10   is represented by the motif Xaa-His-Glu-Phe-Gly-His-Xaa, in
which the two histidine residues coordinate the $Zn^{..}$ in the
active center and the glutamate is involved in bond-breaking
process.

SEQUENCE LISTING

15

(1) GENERAL INFORMATION:
    (i) APPLICANT: Dovey, Harry F.
                   Seubert, Peter
                   Sinha, Sukanto

20   McConlogue, Lisa C.
                   Little, Sheila P.
                   Johnstone, Edward M.
   (ii) TITLE OF INVENTION: Amyloidin Protease and Uses
                         Thereof

25   (iii) NUMBER OF SEQUENCES: 12
   (iv) CORRESPONDENCE ADDRESS:
      (A) ADDRESSEE: Athena Neurosciences, Inc.
      (B) STREET: 800F Gateway Blvd.
      (C) CITY: South San Francisco

30   (D) STATE: California
      (E) COUNTRY: USA
      (F) ZIP: 94080
    (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk

35   (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
   (vi) CURRENT APPLICATION DATA:
      (A) APPLICATION NUMBER: US

40   (B) FILING DATE:
      (C) CLASSIFICATION:
  (viii) ATTORNEY/AGENT INFORMATION:
      (A) NAME: Murphy, Lisabeth Feix
      (B) REGISTRATION NUMBER: 31547

45   (C) REFERENCE/DOCKET NUMBER: 17796-002
   (ix) TELECOMMUNICATION INFORMATION:
      (A) TELEPHONE: (415) 877-0900
      (B) TELEFAX: (415) 877-8370

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(2) INFORMATION FOR SEQ ID NO:1:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 10 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: protein
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
      (v) FRAGMENT TYPE: internal
     (ix) FEATURE:
            (A) NAME/KEY: Modified-site
            (B) LOCATION: 4
            (D) OTHER INFORMATION: /label= Xaa4
                  /note= "Xaa4 can be either Gln or Lys"
     (ix) FEATURE:
            (A) NAME/KEY: Modified-site
            (B) LOCATION: 5
            (D) OTHER INFORMATION: /label= Xaa5
                  /note= "Xaa5 can be either Met or Nle"
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

      Ser Glu Val Xaa Xaa Asp Ala Glu Phe Arg
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(2) INFORMATION FOR SEQ ID NO:2:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 21 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: protein
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
      (v) FRAGMENT TYPE: internal
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

      Arg Val Tyr Asp Gln Val Gly Thr Gln Glu Phe Glu Asp Val Ser Tyr
      1               5                   10                  15

      Glu Ser Thr Leu Lys
                  20

(2) INFORMATION FOR SEQ ID NO:3:
      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 30 amino acids
            (B) TYPE: amino acid
            (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: protein
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
      (v) FRAGMENT TYPE: internal
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

      Val Asp Gln Ala Leu His Thr Gln Thr Asp Ala Asp Pro Ala Glu Glu
      1               5                   10                  15

      Tyr Ala Arg Leu Cys Gln Glu Ile Leu Gly Val Pro Ala Thr
                  20                  25                  30
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(2) INFORMATION FOR SEQ ID NO:4:
      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 26 amino acids
           (B) TYPE: amino acid
           (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: protein
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
      (v) FRAGMENT TYPE: internal
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

          Glu Tyr Phe Pro Val Gln Val Val Thr His Gly Leu Leu Gly Ile Tyr
          1   5   10   15
          Gln Glu Leu Leu Gly Leu Ala Phe His His
                      20   25

(2) INFORMATION FOR SEQ ID NO:5:
      (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 689 amino acids
           (B) TYPE: amino acid
           (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: protein
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

          Met Lys Pro Pro Ala Ala Cys Ala Gly Asp Met Ala Asp Ala Ala Ser
          1   5   10   15

          Pro Cys Ser Val Val Asn Asp Leu Arg Trp Asp Leu Ser Ala Gln Gln
                      20   25   30

          Ile Glu Glu Arg Thr Arg Glu Leu Ile Glu Gln Thr Lys Arg Val Tyr
                      35   40   45

          Asp Gln Val Gly Thr Gln Glu Phe Glu Asp Val Ser Tyr Glu Ser Thr
                      50   55   60

          Leu Lys Ala Leu Ala Asp Val Glu Val Thr Tyr Thr Val Gln Arg Asn
          65   70   75   80

          Ile Leu Asp Phe Pro Gln His Val Ser Pro Ser Lys Asp Ile Arg Thr
                      85   90   95

          Ala Ser Thr Glu Ala Asp Lys Lys Leu Ser Glu Phe Asp Val Glu Met
                      100   105   110

          Ser Met Arg Glu Asp Val Tyr Gln Arg Ile Val Trp Leu Gln Glu Lys
                      115   120   125

          Val Gln Lys Asp Ser Leu Arg Pro Glu Ala Ala Arg Tyr Leu Glu Arg
                      130   135   140

          Leu Ile Lys Leu Gly Arg Arg Asn Gly Leu His Leu Pro Arg Glu Thr
          145   150   155   160

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```
        Gln Glu Asn Ile Lys Arg Ile Lys Lys Leu Ser Leu Leu Cys Ile
                    165             170             175

        Asp Phe Asn Lys Asn Leu Asn Glu Asp Thr Thr Phe Leu Pro Phe Thr
 5                  180             185             190

        Leu Gln Glu Leu Gly Gly Leu Pro Glu Asp Phe Leu Asn Ser Leu Glu
                    195             200             205

        Lys Met Glu Asp Gly Lys Leu Lys Val Thr Leu Lys Tyr Pro His Tyr
10          210             215             220

        Phe Pro Leu Leu Lys Lys Cys His Val Pro Glu Thr Arg Arg Lys Val
        225             230             235             240

15      Glu Glu Ala Phe Asn Cys Arg Cys Lys Glu Glu Asn Cys Ala Ile Leu
                    245             250             255

        Lys Glu Leu Val Thr Leu Arg Ala Gln Lys Ser Arg Leu Leu Gly Phe
                    260             265             270

20      His Thr His Ala Asp Tyr Val Leu Glu Met Asn Met Ala Lys Thr Ser
                    275             280             285

        Gln Thr Val Ala Thr Phe Leu Asp Glu Leu Ala Gln Lys Leu Lys Pro
25          290             295             300

        Leu Gly Glu Gln Glu Arg Ala Val Ile Leu Glu Leu Lys Arg Ala Glu
        305             310             315             320

30      Cys Glu Arg Arg Gly Leu Pro Phe Asp Gly Arg Ile Arg Ala Trp Asp
                    325             330             335

        Met Arg Tyr Tyr Met Asn Gln Val Glu Glu Thr Arg Tyr Cys Val Asp
                    340             345             350

35      Gln Asn Leu Leu Lys Glu Tyr Phe Pro Val Gln Val Val Thr His Gly
                    355             360             365

        Leu Leu Gly Ile Tyr Gln Glu Leu Leu Gly Leu Ala Phe His His Glu
40          370             375             380

        Glu Gly Ala Ser Ala Trp His Glu Asp Val Arg Leu Tyr Thr Ala Arg
        385             390             395             400

45      Asp Ala Ala Ser Gly Glu Val Val Gly Lys Phe Tyr Leu Asp Leu Tyr
                    405             410             415

        Pro Arg Glu Gly Lys Tyr Gly His Ala Ala Cys Phe Gly Leu Gln Pro
                    420             425             430

50      Gly Cys Leu Arg Gln Asp Gly Ser Arg Gln Ile Ala Ile Ala Ala Met
                    435             440             445

        Val Ala Asn Phe Thr Lys Pro Thr Ala Asp Ala Pro Ser Leu Leu Gln
55          450             455             460
```

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```
His Asp Glu Val Glu Thr Tyr Phe His Glu Phe Gly His Val Met His
465             470             475             His     480
```

```
Gln Leu Cys Ser Gln Ala Glu Phe Ala Met Phe Ser Gly Thr His Val
            485             490             495
```

```
Glu Arg Asp Phe Val Glu Ala Pro Ser Gln Met Leu Glu Asn Trp Val
        500             505             510
```

```
Trp Glu Gln Glu Pro Leu Leu Arg Met Ser Arg His Tyr Arg Thr Gly
        515             520             525
```

```
Ser Ala Val Pro Arg Glu Leu Leu Glu Lys Leu Ile Glu Ser Arg Gln
        530             535             540
```

```
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545             550             555             560
```

```
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            565             570             575
```

```
Ala Arg Leu Cys Gln Glu Ile Leu Gly Val Pro Ala Thr Pro Gly Thr
        580             585             590
```

```
Asn Met Pro Ala Thr Phe Gly His Leu Ala Gly Gly Tyr Asp Ala Gln
        595             600             605
```

```
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        610             615             620
```

```
Thr Arg Phe Lys Gln Glu Gly Val Leu Asn Ser Lys Val Gly Met Asp
625             630             635             640
```

```
Tyr Arg Ser Cys Ile Leu Arg Pro Gly Gly Ser Glu Asp Ala Ser Ala
            645             650             655
```

```
Met Leu Arg Arg Phe Leu Gly Arg Asp Pro Lys Gln Asp Ala Phe Leu
        660             665             670
```

```
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```

```
Cys
```

(2) INFORMATION FOR SEQ ID NO:6:
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 2070 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
    (iv) ANTI-SENSE: NO
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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```
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ATCGAGCAGA CCAAGCGCGT GTATGACCAG GTTGGCACCC AGGAGTTTGA GGACGTGTCC     180

TACGAGAGCA CGCTCAAGGC GCTGGCCGAT GTGGAGGTCA CCTACACAGT TCAGAGGAAT     240

ATCCTTGACT TCCCCCAGCA TGTTTCCCCC TCCAAGGACA TCCGGACAGC CAGCACAGAG     300

GCCGACAAGA AGCTCTCTGA GTTCGACGTG GAGATGAGCA TGACGGAGGA CGTGTACCAG     360

AGGATCGTGT GGCTCCAGGA GAAAGTTCAG AAGGACTCAC TGAGCCCCGA GGCTGCGCGG     420

TACCTGGAGC GGCTAATCAA GCTGGGCCGG AGAAATGGGC TTCACCTCCC CAGAGAGACT     480

CAGGAAAACA TCAAACGCAT CAAGAAGAAG CTGAGCCTTC TGTGCATCGA CTTCAACAAG     540

AACCTGAACG AGGACACGAC CTTCCTGCCC TTCACGCTCC AGGAGCTAGG AGGGCTCCCC     600

GAGGACTTTC TGAACTCCCT GGAGAAGATG GAGGACGGCA AGTTGAAGGT CACCCTCAAG     660

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GAGGAGGCCT TCAACTGCCG GTGCAAGGAG GAGAACTGCG CTATCCTCAA GGAGCTGGTG     780

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GAGATGAACA TGGCCAAGAC CAGCCAGACC GTGGCCACCT TCCTAGATGA GCTGGCGCAG     900

AAGCTGAAGC CCCTGGGGGA GCAGGAGCGT GCGGTGATTC TGGAGCTGAA GCCGTGCGGAG     960

TGCGAGCGCC GGGGCCTGCC CTTCGACGGC CGCATCCGTG CCTGGGACAT GCGCTACTAC    1020

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TTCCACCACG AGGAGGGCGC CAGTGCCTGG CATGAGGACG TGCGGCTCTA CACCGCGAGG    1200

GACGCGGCCT CGGGGGAGGT GGTCGGCAAG TTCTACCTGG ACCTGTACCC GCGGGAAGGA    1260

AAGTACGGGC ACGCGGCCTG CTTTGGCCTG CAGCCCGGCT GCCTGCGGCA GGATGGGAGC    1320

CGCCAGATCG CCATCGCGGC CATGGTGGCC AACTTCACCA AGCCCACAGC CGACGCGCCC    1380

TCGCTGCTGC AGCATGACGA GGTGGAGACC TACTTCCATG AGTTCGGCCA CGTGATGCAC    1440

CAGCTCTGCT CCCAGGCGGA GTTCGCCATG TTCAGCGGGA CCCACGTGGA GCGGGACTTT    1500

GTGGAGGCGC CGTCGCAGAT GCTGGAGAAC TGGGTGTGGG AGCAGGAGCC GCTGCTGCGG    1560

ATGTCGCGGC ACTACCGCAC AGGCAGCGCC GTGCCCCGGG AGCTCCTGGA GAAGCTCATT    1620

GAGTCCCGGC AGGCCAACAC AGGCCTCTTC AGCCTGCGCC AGATCGTCCT CGCCAAGGTG    1680

GACCAGGCCC TGCACACGCA GACGGACGCA GACCCCGCCG AGGAGTATGC GCGGCTCTGC    1740
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CAGGAGATCC TCGGGGTCCC GGCCACGCCA GGAACCAACA TGCCTGCAAC CTTCGGCCAT   1800

CTGGCAGGTG GCTACGACGC CCAGTACTAC GGGTACCTGT GGAGCGAGGT GTATTCCATG   1860

GACATGTTCC ACACGCGCTT CAAGCAGGAG GGTGTCCTGA ACAGCAAGGT TGGCATGGAT   1920

TACAGAAGCT GCATCCTGAG ACCCGGCGGT TCCGAGGATG CCAGCGCCAT GCTGAGGCGC   1980

TTCCTGGGCC GTGACCCCAA GCAGGACGCC TTCCTCCTGA GCAAGGGGCT GCAGGTCGGG   2040

GGCTGCGAGC CCGAGCCGCA GGTCTGCTGA   2070

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(2) INFORMATION FOR SEQ ID NO:7:
      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 25 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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(2) INFORMATION FOR SEQ ID NO:8:
      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 25 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: YES
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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(2) INFORMATION FOR SEQ ID NO:9:
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          (A) LENGTH: 27 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE: cDNA
    (iii) HYPOTHETICAL: NO
     (iv) ANTI-SENSE: NO
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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(2) INFORMATION FOR SEQ ID NO:10:
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          (A) LENGTH: 25 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
```

```
                    (D) TOPOLOGY: linear
                (ii) MOLECULE TYPE: cDNA
               (iii) HYPOTHETICAL: NO
                (iv) ANTI-SENSE: NO
 5              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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      (2) INFORMATION FOR SEQ ID NO:11:
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                    (A) LENGTH:  23 base pairs
10                  (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear
                (ii) MOLECULE TYPE: cDNA
               (iii) HYPOTHETICAL: NO
15              (iv) ANTI-SENSE: YES
                (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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             (i) SEQUENCE CHARACTERISTICS:
20                  (A) LENGTH: 23 base pairs
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                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear
                (ii) MOLECULE TYPE: cDNA
25             (iii) HYPOTHETICAL: NO
                (iv) ANTI-SENSE: YES
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                    CAAAGCTTRT GRTTNGCYAA NCC        23

      (2) INFORMATION FOR SEQ ID NO:13:
30           (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 75 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear
35              (ii) MOLECULE TYPE: cDNA
               (iii) HYPOTHETICAL: YES
                (iv) ANTI-SENSE: NO
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           AAGGAGTACT TCCCTGTGCA GGTGGTCACG CACGGGCTGC TGGGCATCTA CCAGGAGCTC    60
40
           CTGGGCCTGG CCTTC                                                   75

      (2) INFORMATION FOR SEQ ID NO:14:
             (i) SEQUENCE CHARACTERISTICS:
45                  (A) LENGTH: 18 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear
                (ii) MOLECULE TYPE: cDNA
50             (iii) HYPOTHETICAL: NO
```

-45-

```
        (iv) ANTI-SENSE: NO
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

            GAAATGCACG TGCCTGAG              18
5

    (2) INFORMATION FOR SEQ ID NO:15:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 17 base pairs
            (B) TYPE: nucleic acid
10          (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: cDNA
       (iii) HYPOTHETICAL: NO
        (iv) ANTI-SENSE: YES
15      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

            CCAGGACATA GTCGGCG              17


    (2) INFORMATION FOR SEQ ID NO:16:
        (i) SEQUENCE CHARACTERISTICS:
20          (A) LENGTH: 164 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
25      (ii) MOLECULE TYPE: cDNA
       (iii) HYPOTHETICAL: NO
        (iv) ANTI-SENSE: NO
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

30     GAAATGCACG TGCCTGAGAC CAGGAGGAAA GTGGAGGAGG CCTTCAACTG CCGGTGCAAG     60

       CCGGTGCAAG GAGGAGAACT GCGCTATCCT CAAGGAGCTG GTGACGCTGC GGGCCCAGAA    120

       GTCCCGCCTG CTGGGGTTCC ACACGCACGC CGACTATGTC CTGG                     164
35


    (2) INFORMATION FOR SEQ ID NO:17:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 18 base pairs
40          (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: cDNA
       (iii) HYPOTHETICAL: NO
45      (iv) ANTI-SENSE: YES
        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

            ACTTTCCTCC TGGTCTCA              18


50  (2) INFORMATION FOR SEQ ID NO:18:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 18 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
55          (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE: cDNA
       (iii) HYPOTHETICAL: NO
```

(iv) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

        GGAGAAGCTC ATTGAGTC   18

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We claim:

1.   Human amyloidin protease, capable of cleaving the
Met-Asp bond in the substrate: acetyl-Ser-Glu-Val-Lys-
Met-Asp-Ala-Glu-Phe-Arg (Seq ID No:1), substantially free of
5   natural contaminants.

2.   Human amyloidin protease, which has a molecular
weight of about 80,000 daltons as determined by SDS-
polyacrylamide gel electrophoresis.

3.   The human amyloidin protease of claim 1, having the
10   primary sequence (Seq ID No: 5):

```
Met Lys Pro Pro Ala Ala Cys Ala Gly Asp Met Ala Asp Ala Ala Ser
 1           5               10              15

Pro Cys Ser Val Val Asn Asp Leu Arg Trp Asp Leu Ser Ala Gln Gln
             20              25              30

Ile Glu Glu Arg Thr Arg Glu Leu Ile Glu Gln Thr Lys Arg Val Tyr
         35              40              45

Asp Gln Val Gly Thr Gln Glu Phe Glu Asp Val Ser Tyr Glu Ser Thr
         50              55              60

Leu Lys Ala Leu Ala Asp Val Glu Val Thr Tyr Thr Val Gln Arg Asn
65              70              75              80

Ile Leu Asp Phe Pro Gln His Val Ser Pro Ser Lys Asp Ile Arg Thr
             85              90              95

Ala Ser Thr Glu Ala Asp Lys Lys Leu Ser Glu Phe Asp Val Glu Met
             100             105             110

Ser Met Arg Glu Asp Val Tyr Gln Arg Ile Val Trp Leu Gln Glu Lys
         116             120             125

Val Gln Lys Asp Ser Leu Arg Pro Glu Ala Ala Arg Tyr Leu Glu Arg
         130             135             140

Leu Ile Lys Leu Gly Arg Arg Asn Gly Leu His Leu Pro Arg Glu Thr
145             150             155             160

Gln Glu Asn Ile Lys Arg Ile Lys Lys Lys Leu Ser Leu Leu Cys Ile
                165             170             175

Asp Phe Asn Lys Asn Leu Asn Glu Asp Thr Thr Phe Leu Pro Phe Thr
             180             185             190

Leu Gln Glu Leu Gly Gly Leu Pro Glu Asp Phe Leu Asn Ser Leu Glu
             195             200             205
```

```
        Lys Met Glu Asp Gly Lys Leu Lys Val Thr Leu Lys Tyr Pro His Tyr
            210             215             220

 5      Phe Pro Leu Leu Lys Lys Cys His Val Pro Glu Thr Arg Arg Lys Val
        225             230             235             240

        Glu Glu Ala Phe Asn Cys Arg Cys Lys Glu Glu Asn Cys Ala Ile Leu
                    245             250             255

10      Lys Glu Leu Val Thr Leu Arg Ala Gln Lys Ser Arg Leu Leu Gly Phe
                    260             265             270

        His Thr His Ala Asp Tyr Val Leu Glu Met Asn Met Ala Lys Thr Ser
                    275             280             285

15
        Gln Thr Val Ala Thr Phe Leu Asp Glu Leu Ala Gln Lys Leu Lys Pro
                    290             295             300

20      Leu Gly Glu Gln Glu Arg Ala Val Ile Leu Glu Leu Lys Arg Ala Glu
        305             310             315             320

        Cys Glu Arg Arg Gly Leu Pro Phe Asp Gly Arg Ile Arg Ala Trp Asp
                    325             330             335

25      Met Arg Tyr Tyr Met Asn Gln Val Glu Glu Thr Arg Tyr Cys Val Asp
                    340             345             350

        Gln Asn Leu Leu Lys Glu Tyr Phe Pro Val Gln Val Val Thr His Gly
                    355             360             365

30      Leu Leu Gly Ile Tyr Gln Glu Leu Leu Gly Leu Ala Phe His His Glu
        370             375             380

        Glu Gly Ala Ser Ala Trp His Glu Asp Val Arg Leu Tyr Thr Ala Arg
35      385             390             395             400

        Asp Ala Ala Ser Gly Glu Val Val Gly Lys Phe Tyr Leu Asp Leu Tyr
                    405             410             415

40      Pro Arg Glu Gly Lys Tyr Gly His Ala Ala Cys Phe Gly Leu Gln Pro
                    420             425             430

        Gly Cys Leu Arg Gln Asp Gly Ser Arg Gln Ile Ala Ile Ala Ala Met
                    435             440             445

45
        Val Ala Asn Phe Thr Lys Pro Thr Ala Asp Ala Pro Ser Leu Leu Gln
            450             455             460

        His Asp Glu Val Glu Thr Tyr Phe His Glu Phe Gly His Val Met His
50      465             470             475             480

        Gln Leu Cys Ser Gln Ala Glu Phe Ala Met Phe Ser Gly Thr His Val
                    485             490             495

55      Glu Arg Asp Phe Val Glu Ala Pro Ser Gln Met Leu Glu Asn Trp Val
                    500             505             510
```

Trp Glu Gln Glu Pro Leu Leu Arg Met Ser Arg His Tyr Arg Thr Gly
        515   520   525

Ser Ala Val Pro Arg Glu Leu Leu Glu Lys Leu Ile Glu Ser Arg Gln
        530   535   540

Ala Asn Thr Gly Leu Phe Ser Leu Arg Gln Ile Val Leu Ala Lys Val
545   550   555   560

Asp Gln Ala Leu His Thr Gln Thr Asp Ala Asp Pro Ala Glu Glu Tyr
                565   570   575

Ala Arg Leu Cys Gln Glu Ile Leu Gly Val Pro Ala Thr Pro Gly Thr
        580   585   590

Asn Met Pro Ala Thr Phe Gly His Leu Ala Gly Gly Tyr Asp Ala Gln
        595   600   605

Tyr Tyr Gly Tyr Leu Trp Ser Glu Val Tyr Ser Met Asp Met Phe His
        610   615   620

Thr Arg Phe Lys Gln Glu Gly Val Leu Asn Ser Lys Val Gly Met Asp
625   630   635   640

Tyr Arg Ser Cys Ile Leu Arg Pro Gly Gly Ser Glu Asp Ala Ser Ala
                645   650   655

Met Leu Arg Arg Phe Leu Gly Arg Asp Pro Lys Gln Asp Ala Phe Leu
        660   665   670

Leu Ser Lys Gly Leu Gln Val Gly Gly Cys Glu Pro Glu Pro Gln Val
        675   680   685

Cys.

4.   The human amyloidin protease of claim 1, having an
endopeptidic protease activity capable of cleaving the Asp-Ala
and the Ala-Glu peptide bonds in the peptide.

5.   The human amyloidin protease of claim 1, which is a
metalloprotease.

6.   A method for purifying human amyloidin protease from
cells, said method comprising:
    a)   disrupting human cells to form an aqueous extract and
an insoluble fraction;
    b)   using chromatographic fractionation on the aqueous
extract to produce an enriched fraction having amyloidin
protease activity;

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    c)   binding the enriched fraction of (b) to a hydrophobic interaction chromatography matrix;

    d)   eluting the bound protease activity by gradient fractionation to form an eluate; and

    e)   selecting for fractions from the eluate having the ability to hydrolyze an internal Met-Asp peptide bond in an amyloid-like peptide substrate.

7.   The method of claim 6, wherein the human cells are present in brain tissue, and the brain tissue is homogenized in the presence of a buffer having a pH of about 6 to 8.

8.   The method of claim 6, wherein the cells are erythrocytes and cells disruption is by osmotic cell lysis.

9.   The method of claim 6, wherein the chromatographic fractionation is selected from the group consisting of ion exchange chromatography, dye ligand chromatography, size exclusion chromatography, Chromatofocusing®, hydroxyapatite chromatography or a combination thereof.

10.   The method of claim 9, wherein the ion exchange chromatography employs an anion exchange resin for removal of neutral or basic proteins in the aqueous extract to produce an enriched fraction.

11.   The method of claim 9, wherein the enriched fraction is applied to a hydrophobic interaction chromatography matrix in a high salt buffer.

12.   The method of claim 11, wherein the high salt buffer is approximately 1 M ammonium sulfate.

13.   The method of claim 11, wherein the hydrophobic interaction chromatography matrix is a Phenyl-TSK HPLC column.

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14. The method of claim 12, wherein gradient fractionation of step (d) consists of a 1 to 0 M descending gradient of ammonium sulfate.

15. The method of claim 14 which further comprises after step (d), collecting in a pH 6 to 8 buffer, the amyloidin protease.

16. The method of claim 9, wherein the chromatographic fractionation is sequentionally performed using anion exchange, hydroxyapatite and Chromatofocusing® chromatography.

17. The method of claim 9, wherein the chromatographic fractionation is sequentionally performed using anion exchang and Chromatofocusing® chromatography.

18. An antibody obtained by means of an immune response in a mammal having been exposed to the human amyloidin protease of claim 1.

19. An antibody obtained by means of an immune response in a mammal having been exposed to a peptide fragment of the protein of claim 1, said fragment corresponding substantially to the sequence Val-Tyr-Asp-Gln-Val-Gly-Thr-Gln-Glu-Phe-Glu-Asp-Val-Ser-Tyr-Glu-Ser-Thr-Leu-Lys (Seq ID No:2).

20. The antibody of claim 18, wherein the antibody is a polyclonal antibody.

21. The antibody of claim 18, wherein the antibody is a monoclonal antibody.

22. An immunogenic peptide fragment of the protease of claim 1, wherein the peptide fragment is about 10 amino acids in length.

23. The immunogenic p ptide fragment of claim 22, wherein the p ptide fragment is about 20 amino acids.

24. A method for the identification of an inhibitor of
the proteas of claim 1, said method comprising:
a) combining the prot ase with a compound of interest
under conditions sufficient to form a mixture comprising a
5   complex of the protease and the compound of interest;
b) introducing the mixture to an amyloid-like substrate
under conditions sufficient to cleave the Met-Asp bond in said
substrate; and
c) monitoring whether cleavage of the substrate has
10   occurred.

25. The method of claim 24, wherein the protease and the
compound of interest are initially combined in the presence of
the substrate.

26. A method for the identification of an inhibitor of a
15   protease capable of cleaving an endopeptidic Met-Asp bond in
an amyloid-like substrate, said method comprising:
a) combining the protease with a compound of interest
under conditions sufficient to cleave the Met-Asp bond of the
amyloid-like substrate; and
20   b) monitoring whether cleavage of the substrate has
occurred.

27. The method of claim 26, wherein the protease is
human amyloidin protease.

28. The method of claim 26, wherein the protease is a
25   mammalian chymase.

29. The method of claim 26, wherein the mammalian
chymase is selected from the group consisting of human skin
chymase and rat mast cell proteases I and II.

30. The method of claim 26, wh rein the amyloid-like
30   substrate corresponds substantially to th peptide:
acetyl-Ser-Glu-Val-Xaa,-Xaa,-Asp-Ala-Glu-Phe-Arg

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wherein Xaa, is Gln or Lys and Xaa, is Met or Nle (Seq ID No:1).

31.   The method of claim 26 wherein the amyloid-like substrate is a fragment of the amyloid precursor protein.

5   32.   The method of claim 26, wherein the amyloid-like substrate is labeled.

33.   The method of claim 32, wherein the label comprises a signal means and a capture means.

34.   The method of claim 33, wherein the capture means is
10   a specific binding partner.

35.   The method of claim 33, wherein the signal means is capable of emitting radiation.

36.   A DNA sequence encoding the human amyloidin protease of claim 1.

15   37.   The DNA sequence of claim 36, comprising the nucleotide sequence (Seq ID No:6):

```
        ATGAAGCCCC CCGCAGCCTG TGCAGGAGAC ATGGCGGACG CAGCATCTCC GTGCTCTGTG      60

        GTAAACGACC TGCGGTGGGA CCTGAGTGCC CAGCAGATAG AGGAGCGCAC CAGGGAGCTC     120
20
        ATCGAGCAGA CCAAGCGCGT GTATGACCAG GTTGGCACCC AGGAGTTTGA GGACGTGTCC     180

        TACGAGAGCA CGCTCAAGGC GCTGGCCGAT GTGGAGGTCA CCTACACAGT TCAGAGGAAT     240

25      ATCCTTGACT TCCCCCAGCA TGTTTCCCCC TCCAAGGACA TCCGGACAGC CAGCACAGAG     300

        GCCGACAAGA AGCTCTCTGA GTTCGACGTG GAGATGAGCA TGAGGGAGGA CGTGTACCAG     360

        AGGATCGTGT GGCTCCAGGA GAAAGTTCAG AAGGACTCAC TGAGGCCCGA GGCTGCGCGG     420
30
        TACCTGGAGC GGCTAATCAA GCTGGGCCGG AGAAATGGGC TTCACCTCCT CAGAGAGACT     480

        CAGGAAAACA TCAAACGCAT CAAGAAGAAG CTGAGCCTTC TGTGCATCGA CTTCAACAAG     540

35      AACCTGAACG AGGACACGAC CTTCCTGCCC TTCACGCTCC AGGAGCTAGG AGGGCTCCCC     600

        GAGGACTTTC TGAACTCCCT GGAGAAGATG GAGGACGGCA AGTTGAAGGT CACCCTCAAG     660

        TACCCCCATT ACTTCCCCCT CCTGAAGAAA TGCCACGTGC CTGAGACCAG GAGGAAAGTG     720
```

```
        GAGGAGGCCT TCAACTGCCG GTGCAAGGAG GAGAACTGCG CTATCCTCAA CGAGCTGGTG    780

        ACGCTGCGGG CCCAGAAGTC CCGCCTGCTG GGGTTCCACA CGCACGCCGA CTATGTCCTG    840

   5    GAGATGAACA TGGCCAAGAC CAGCCAGACC GTGGCCACCT TCCTAGATGA GCTGGCGCAG    900

        AAGCTGAAGC CCCTGGGGGA GCAGGAGCGT GCGGTGATTC TGGAGCTGAA CCGTGCGGAG    960

  10    TGCGAGCGCC GGGGCCTGCC CTTCGACGGC CGCATCCGTG CCTGGGACAT GCGCTACTAC   1020

        ATGAACCAGG TGGAGGAGAC GCGCTACTGC GTGGACCAGA ACCTGCTCAA CGAGTACTTC   1080

        CCCGTGCAGG TGGTCACGCA CGGGCTGCTG GGCATCTACC AGGAGCTCCT GGGGCTGGCC   1140

  15    TTCCACCACG AGGAGGGCGC CAGTGCCTGG CATGAGGACG TGCGGCTCTA CACCGCGAGG   1200

        GACGCGGCCT CGGGGGAGGT GGTCGGCAAG TTCTACCTGG ACCTGTACCC GCGGGAAGGA   1260

  20    AAGTACGGGC ACGCGGCCTG CTTTGGCCTG CAGCCCGGCT GCCTGCGGCA GGATGGGAGC   1320

        CGCCAGATCG CCATCGCGGC CATGGTGGCC AACTTCACCA AGCCCACAGC CGACGCGCCC   1380

        TCGCTGCTGC AGCATGACGA GGTGGAGACC TACTTCCATG AGTTTGGCCA CGTGATGCAC   1440

  25    CAGCTCTGCT CCCAGGCGGA GTTCGCCATG TTCAGCGGGA CCCACGTGGA CGGGACTTT    1500

        GTGGAGGCGC CGTCGCAGAT GCTGGAGAAC TGGGTGTGGG AGCAGGAGCC GCTGCTGCGG   1560

  30    ATGTCGCGGC ACTACCGCAC AGGCAGCGCC GTGCCCCGGG AGCTCCTGGA GAAGCTCATT   1620

        GAGTCCCGGC AGGCCAACAC AGGCCTCTTC AGCCTGCGCC AGATCGTCCT CGCCAAGGTG   1680

        GACCAGGCCC TGCACACGCA GACGGACGCA GACCCCGCCG AGGAGTATGC CCGGCTCTGC   1740

  35    CAGGAGATCC TCGGGGTCCC GGCCACGCCA GGAACCAACA TGCCTGCAAC CTTCGGCCAT   1800

        CTGGCAGGTG GCTACGACGC CCAGTACTAC GGGTACCTGT GGAGCGAGGT GTATTCCATG   1860

  40    GACATGTTCC ACACGCGCTT CAAGCAGGAG GGTGTCCTGA ACAGCAAGGT TGGCATGGAT   1920

        TACAGAAGCT GCATCCTGAG ACCCGGCGGT TCCGAGGATG CCAGCGCCAT GCTGAGGCGC   1980

        TTCCTGGGCC GTGACCCCAA GCAGGACGCC TTCCTCCTGA GCAAGGGGCT GCAGGTCGGG   2040

  45    GGCTGCGAGC CCGAGCCGCA GGTCTGCTGA.                                     2070
```

38. A cell transformed with a recombinant vector comprising the DNA sequence of claim 36, said transformed cell capable of producing human amyloidin protease.
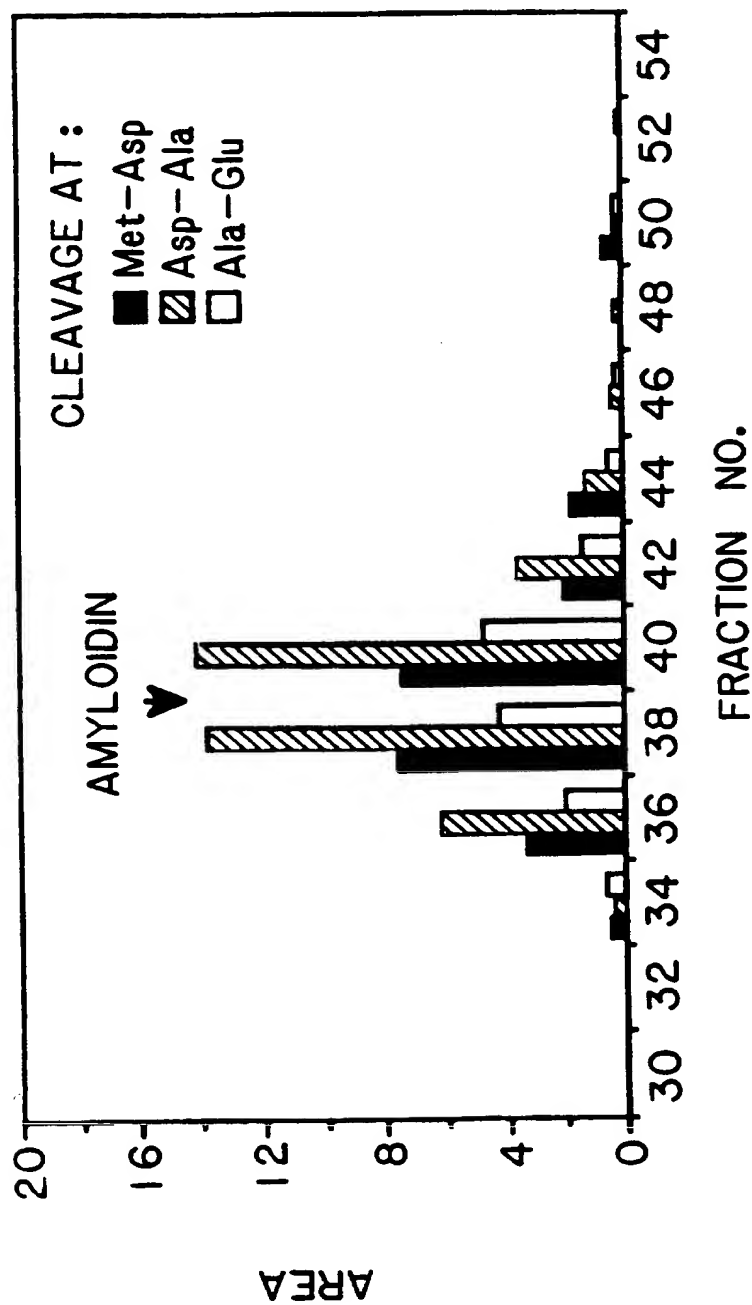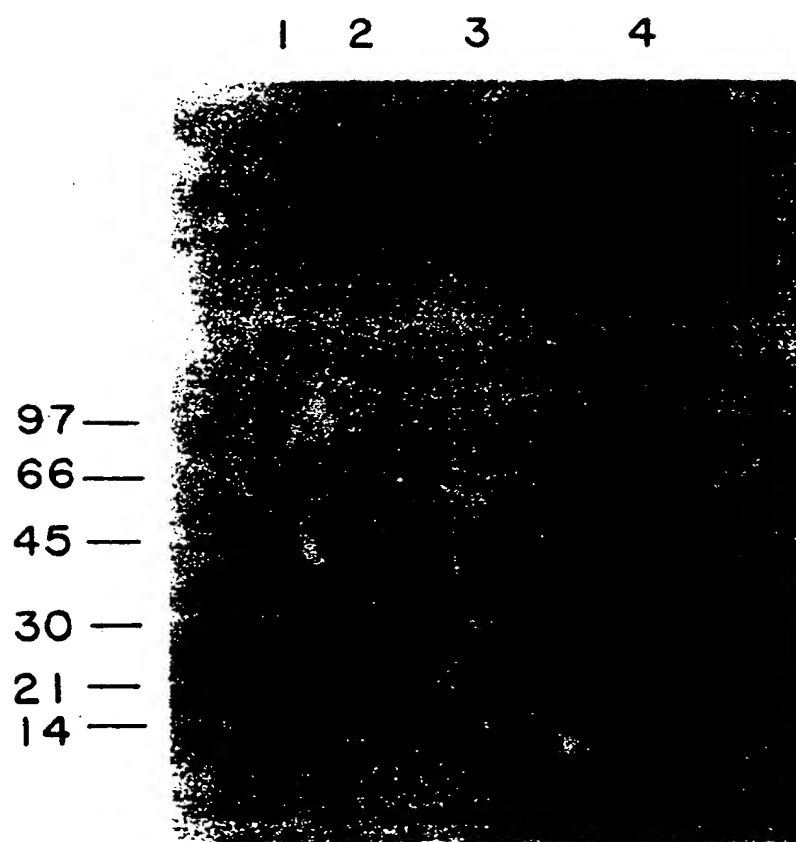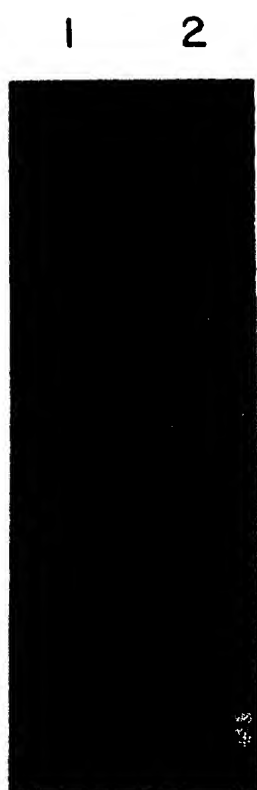
1/4

FIG. 1

FIG. 2

FIG. 3A   FIG. 3B

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FIG. 4

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATI N F SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 9/64, 15/57;C12Q 1/37; C07K 7/00, 15/28
U.S.Cl.: 435/23, 226, 252.3; 530/328, 326, 387; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched 7	
Classification System	Classification Symbols
U.S.	435/23, 219, 226, 252.3; 530/328, 326, 387; 536/27

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

Computer Search - CA, Sequence and APS

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X,P Y	Biochem. Biophys. Res. Comm., Volume 174, Number 2, issued 31 January 1991, C.R. Abraham, et al., "A Calcium-Activated Protease From Alzheimer's Disease Brain Cleaves At The N-Terminus Of The Amyloid B-Protein", pages 790-796, see all.	1,5 18,20-27, 30-36, 38
X,P Y	J. Cell. Biochem., Volume supplement 15G, issued 06-25 April 1991, C.R. Abraham, et al., "Studies On The Proteolytic Degradation Of The Amyloid Precursor Protein In Alzheimer's Disease", page 115, see the abstract CH 024.	1,5 18,20-27, 30-36, 38
X Y	Neurobiology Of Aging, Volume 11, issued 08 May 1990, C.R. Abraham et al., "Studies On A Brain Protease That Cleaves At The N-Terminus Of B-Protein-Related Synthetic Peptides", page 303, see the abstract 207.	1-5 18,20-27, 30-36, 38

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 January 1992	**19 MAR 1992**
International Searching Authority	Signature of Authorized Officer
ISA/US	Charles Patterson	8P

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X / Y	Soc. Neuroscience Abst., Volume 15, issued 1989, C.R. Abraham, et al., "Proteolytic Processing of B-Protein Precursor-Related Synthetic Peptides", see page 648, the abstract 262.7.	1,5 18,20-27, 30-36, 38

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers   . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers   . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____ , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-17 and 22-35, drawn to a protease, method of preparation and method of use.
II. Claims 18-21, drawn to an antibody.
III. Claims 36-38, drawn to a DNA sequence.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2) (Rev. 11-87)

EUROPEAN PATENT APPLICATION

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Declaration under Rule 28(4) EPC (expert solution)

(54) Protease and related DNA compounds.

(57)   This invention provides an amyloid precursor protein-cleaving protein and related nucleic acid compounds. The invention also provides methods, materials and assays. The compounds of this invention will further the characterization of neurological diseases such as Alzheimer's disease and Down's syndrome.

EP 0 576 152 A1

A peptide of 42 to 43 residues known as the β-amyloid peptide (β/A4) has been implicated in Alzheimer's disease and Down's syndrome. Researchers hypothesize that abnormal accumulation of this 4 kilodalton (kd) protein in the brain is due to cleavage of a larger precursor protein, called amyloid precursor protein (APP). Normal cleavage of APP occurs within the A4 region, indicating that an alternate cleavage event occurs when the normal full length is generated. The amino terminal residue of β/A4 is most often an aspartic acid (Asp), indicating that a protease which cleaves between the methionine (Met) at position 596 [Met$_{596}$ using the numbering system according to J. Kang, et al., Nature 325:733 (1987).] and Asp$_{597}$ of APP would generate amyloid. Therefore, proteases which cleave the APP so as to generate β/A4 are important tools for characterizing Alzheimer's disease and Down's syndrome.

In the past, researchers have attempted to characterize the abnormal cleavage event through the use of classical protein purification techniques. These investigations have resulted in reports of a partially purified 68 kilodalton protease which cleaves at a Met-Asp bond of a synthetic peptide. C. Abraham, et al., Neurobiology of Aging 11A:303 (1990). In 1991, Abraham and co-workers, compared the cleavage pattern of the 68 kd protease with known serine proteases. C. Abraham, et al., Biochemical and Biophysical Research Communications, 174:790 (1991). Subsequently, the same researchers reported that the activity seen in the prior studies was actually the action of two independent proteases. One was identified as a calcium-dependent serine protease and the other a cysteine metalloprotease. C. Abraham, et al., Journal of Cellular Biochemistry, 15:115 (1991); C. Abraham, et al., Journal of Neurochemistry, 57:5109 (1991). No structure or characterization of these proteases was disclosed.

The present invention provides a new enzyme which is structurally different from those previously described and which will cleave APP to generate amyloidogenic fragments of the size expected of a Met$_{596}$-Asp$_{597}$ cleavage. Thus, the new enzyme is very useful in furthering the characterization of Alzheimer's disease and Down's syndrome. Moreover, use of the invention may result in treatments for these or other related diseases.

To date there has been no satisfactory means of diagnosing Alzheimer's disease in a person until the dementia completely manifests itself. Confirmation of the dementia as having arisen from Alzheimer's disease requires a post-mortem examination of the brain of the afflicted patient. The instant invention provides a means of determining those patients having Alzheimer's disease or a propensity of developing Alzheimer's disease while such patients are still alive.

For purposes of clarity and as an aid in understanding the invention, as disclosed and claimed herein, the following items are defined below.

"293 cells" refers to a widely available transformed human priamry embryonal kidney cell line, as described in F.L. Graham, et al., Journal of General Virology, 36:59-72 (1977). This cell line may be obtained, for example, from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 10852-1776 (ATCC), under the accession number ATCC CRL 1573.

"AV12 cells" refers to another widely available cell line which may be obtained from the ATCC under the accession number ATCC CRL 9595.

"Amyloidogenic fragment" - An APP fragment comprising the β/A4 peptide.

"Functional compound of SEQ ID NO:1" - A compound comprising SEQ ID NO:1 which is capable of cleaving APP.

"Kunitz-like domain" - A protease inhibitor similar to soybean trypsin inhibitor or a nucleic acid sequence encoding a protease inhibitor which is similar to the soybean trypsin inhibitor. For example, the Kunitz Protease Inhibitor (KPI) region of APP as described in P. Ponte, et al., Nature 331:525 (1988), or R.E. Tanzi, et al., Nature,331:528 (1988), or N. Kitaguchi, et al., Nature, 331:530 (1988) is a Kunitz-like domain.

"pRc/Zyme" - A modified pRc/CMV eukaryotic expression vector, the pRc/CMV vector being available commerically (Invitrogen Corporation, 3985 Sorrento -Valley Blvd., Suite B, San Diego, California 92121). The plasmid pRc/Zyme comprises a human cytomegalovirus promoter and enhancer, a bovine growth hormone polyadenylation signal, a neomycin resistance gene, a beta-lactamase gene useful as an ampicillin resistance marker in E. coli, and many other features as described in the 1991 Invitrogen Catalog, page 29, as well as a NotI/SalI insert of 1451 base pairs which contains an entire Zyme coding region.

"pSZyme" - A modified E.coli cloning vector pSPORT-1™ [described in E.Y. Chen, et al., DNA, 4:165 (1985)], the plasmid pSPORT-1™ being commercially available (Gibco-BRL, 8400 Helgerman Court, Gaithersburg, Maryland 20877). This plasmid contains an origin of replication from a pUC vector, this plasmid being described in C. Yanisch-Perron, et al., Gene, 33:103-119 (1985); the beta-lactamase gene which confers ampicillin resistance; a NotI/SalI insert of 1451 base pairs which contains an entire coding region of Zyme; as well as other features.

"Part of SEQ ID NO:1" - At least 6 consecutive amino acid residues of SEQ ID NO:1.

"mRNA" - ribonucleic acid (RNA) which has been transcribed either in vivo or in vitro, including, for example, RNA transcripts prepared in vitro by transcription of coding sequences of DNA by RNA polymerase.

"SEQ ID NO:1 or a functional equivalent thereof"
- SEQ ID NO:1 or a conservative alteration of the amino
acid sequence of SEQ ID NO:1, wherein the conservative
alteration results in a compound which exhibits
substantially the same biological, biochemical, chemical,
physical and structural qualities of SEQ ID:1.

"SEQ ID NO:3" - The DNA sequence ATG GCT GGC GGC
ATC ATA GTC AGG G.

"SEQ ID NO:4" - The DNA sequence AAC CGA ATC TTC
AGG TCT TCC TGG GG.

"SEQ ID NO:5" - The DNA sequence TCG CTC TCT CCT
GGG GAC ACA GA.

"SEQ ID NO:6" -The DNA sequence CCA GGT GCT ATT CCA
TGT ATG TCA TAG.

"SEQ ID NO:7" -The DNA sequence TCT GTG TCC CCA GGA
GAG AGC GA.

"SEQ ID NO:8" -The DNA sequence ATA GTG AAG CTG TCT
TCT CAA T.

"Transfection" - any transfer of nucleic acid into a host cell, with or without integration of said nucleic acid
into genome of said host cell.

"Zyme" - the amino acid sequence SEQ ID NO:1 or a functional equivalent thereof.

"Zyme-related band configuration" - One of two band configurations chosen from two band configurations
of a herein disclosed restriction fragment polymorphism. One pattern displays a 2400 base pair band, but no
2500 base pair band. The other pattern displays a 2500 band, but no 2400 base pair band.

The present invention provides amino acid compounds which comprise the amino acid sequence

```
Met Lys Lys Leu Met Val Val Leu Ser Leu Ile Ala Ala Ala Trp Ala
1               5               10              15

Glu Glu Gln Asn Lys Leu Val His Gly Gly Pro Cys Asp Lys Thr Ser
            20              25              30
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His Pro Tyr Gln Ala Ala Leu Tyr Thr Ser Gly His Leu Leu Cys Gly
        35                  40              45

Gly Val Leu Ile His Pro Leu Trp Val Leu Thr Ala Ala His Cys Lys
    50              55                  60

Lys Pro Asn Leu Gln Val Phe Leu Gly Lys His Asn Leu Arg Gln Arg
65                  70              75                      80

Glu Ser Ser Gln Glu Gln Ser Ser Val Val Arg Ala Val Ile His Pro
            85                  90                      95

Asp Tyr Asp Ala Ala Ser His Asp Gln Asp Ile Met Leu Leu Arg Leu
            100             105                 110

Ala Arg Pro Ala Lys Leu Ser Glu Leu Ile Gln Pro Leu Pro Leu Glu
        115                 120                 125

Arg Asp Cys Ser Ala Asn Thr Thr Ser Cys His Ile Leu Gly Trp Gly
    130                 135                 140

Lys Thr Ala Asp Gly Asp Phe Pro Asp Thr Ile Gln Cys Ala Tyr Ile
145                 150             155                     160

His Leu Val Ser Arg Glu Glu Cys Glu His Ala Tyr Pro Gly Gln Ile
            165                 170                 175

Thr Gln Asn Met Leu Cys Ala Gly Asp Glu Lys Tyr Gly Lys Asp Ser
            180                 185                 190

Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Gly Asp His Leu Arg
        195                 200                 205

Gly Leu Val Ser Trp Gly Asn Ile Pro Cys Gly Ser Lys Glu Lys Pro
    210                 215                 220

Gly Val Tyr Thr Asn Val Cys Arg Tyr Thr Asn Trp Ile Gln Lys Thr
225                 230                 235                 240

Ile Gln Ala Lys.
            244
```

hereinafter defined as SEQ ID NO:1, or a functional equivalent thereof. In particular, the amino acid compound which is SEQ ID NO:1 is preferred.

Those in the art will recognize that some alterations of SEQ ID NO:1 will fail to change the function of the amino acid compound. For instance, some hydrophobic amino acids may be exchanged for other hydrophobic amino acids, amino acids with similar side chains may be interchanged, basic amino acids may be interchanged with other basic amino acids, acidic amino acids may be interchanged with other acidic amino acids, small amino acids may be interchanged with other small amino acids or various other conservative changes may be made. Those altered amino acid compounds which confer substantially the same function in substantially the same manner as the exemplified amino acid compound are also encompassed within the present invention.

Artisans will also recognize that this protein can be synthesized by a number of different methods. All of the amino acid compounds of the invention can be made by chemical methods well known in the art, including solid phase peptide synthesis or recombinant methods. Both methods are described in U.S. Patent 4,617,149. Recombinant methods are preferred if a high yield is desired. A general method for the construction of any desired DNA sequence is provided in Brown, et al., Methods in Enzymology, 68:109 (1979).

Other routes of production are well known. Expression in eucaryotic cells can be achieved via SEQ ID NO:2, described infra. For example, the amino acid compounds can be produced in eucaryotic cells using simian virus 40, cytomegalovirus, or mouse mammary tumor virus-derived expression vectors comprising DNA which encodes SEQ ID NO:1. As is well known in the art, some viruses are also appropriate vectors. For ex-

ample, the adenovirus, the vaccinia virus, the herpes virus, the baculovirus, and the rous sarcoma virus are useful. Such a method is described in U.S. Patent 4,775,624. Several alternate methods of expression are described in J. Sambrook, et al., Molecular Cloning: A Laboratory Manual, Chapters 16 and 17 (1989).

In another embodiment, the present invention encompasses nucleic acid compounds which comprise nucleic acid sequences encoding SEQ ID NO:1. As skilled artisans recognize, the amino acid compounds of the invention can be encoded by a multitude of different nucleic acid sequences due to the degeneracy of the genetic code, wherein most of the amino acids are encoded by more than one nucleic acid triplet. Because these alternate nucleic acid sequences would encode the same amino acid sequences, the present invention further comprises these alternate nucleic acid sequences. Preferably, the nucleic acid compound is DNA, sense or antisense mRNA. A most preferred embodiment of a DNA compound which encodes Zyme has this sequence:

ATGAAGAAGC	TGATGGTGGT	GCTGAGTCTG	ATTGCTGCAG	CCTGGGCAGA	50
GGAGCAGAAT	AAGTTGGTGC	ATGGCGGACC	CTGCGACAAG	ACATCTCACC	100
CCTACCAAGC	TGCCCTCTAC	ACCTCGGGCC	ACTTGCTCTG	TGGTGGGGTC	150
CTTATCCATC	CACTGTGGGT	CCTCACAGCT	GCCCACTGCA	AAAAACCGAA	200
TCTTCAGGTC	TTCCTGGGGA	AGCATAACCT	TCGGCAAAGG	GAGAGTTCCC	250
AGGAGCAGAG	TTCTGTTGTC	CGGGCTGTGA	TCCACCCTGA	CTATGATGCC	300
GCCAGCCATG	ACCAGGACAT	CATGCTGTTG	CGCCTGGCAC	GCCCAGCCAA	350
ACTCTCTGAA	CTCATCCAGC	CCCTTCCCCT	GGAGAGGGAC	TGCTCAGCCA	400
ACACCACCAG	CTGCCACATC	CTGGGCTGGG	GCAAGACAGC	AGATGGTGAT	450
TTCCCTGACA	CCATCCAGTG	TGCATACATC	CACCTGGTGT	CCCGTGAGGA	500
GTGTGAGCAT	GCCTACCCTG	GCCAGATCAC	CCAGAACATG	TTGTGTGCTG	550
GGGATGAGAA	GTACGGGAAG	GATTCCTGCC	AGGGTGATTC	TGGGGGTCCG	600
CTGGTATGTG	GAGACCACCT	CCGAGGCCTT	GTGTCATGGG	GTAACATCCC	650
CTGTGGATCA	AAGGAGAAGC	CAGGAGTCTA	CACCAACGTC	TGCAGATACA	700
CGAACTGGAT	CCAAAAAACC	ATTCAGGCCA	AG		732

which is hereinafter defined as SEQ ID NO:2. However, also preferred are those nucleic acid compounds which are sense and antisense mRNA.

Also provided by the present invention are nucleic acid vectors comprising nucleic acids which encode SEQ ID NO:1 or a functional equivalent thereof. The preferred nucleic acid vectors are those which are DNA. Most preferred are DNA vectors which comprise the DNA sequence which is SEQ ID NO:2. An especially preferred DNA vector is the plasmid pSZyme.

E. coli/pSZyme, which contains a cloning vector comprising SEQ ID NO:2, was deposited and made part of the stock culture collection of the Northern Regional Research Laboratories (NRRL), Agricultural Research Service, U.S. Department of Agriculture, Peoria, Illinois, 61604 on April 29, 1992, under the accession number NRRL B-18971. SEQ ID NO:2 can be isolated from the plasmid, for example, as a 1451 base pair NotI/SalI restriction fragment. Other fragments are useful in obtaining SEQ ID NO:2.

Additionally, the DNA sequences can be synthesized using commercially available automated DNA synthesizers, such as the ABS (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404) 380B DNA synthesizer. The DNA sequences can also be generated by the polymerase chain reaction (PCR) as described in U.S. Patent No. 4,889,818.

Restriction fragments of these vectors are also provided. The preferred fragments are the 1451 base pair NotI/SalI restriction fragment, the 803 base pair BsrBI/Esp3I restriction fragment and the 815 base pair EcoNI/BfaI restriction fragment of pSZyme.

Moreover, DNA vectors of the present invention preferably comprise a promoter positioned to drive expression of SEQ ID NO:2, or a functional equivalent thereof. Those vectors wherein said promoter functions in human embryonic kidney cells (293 cells), AV12 cells, yeast cells, or Escherichia coli cells are preferred. The DNA expression vector most preferred is plasmid pRc/Zyme.

The plasmid pSZyme, isolatable from E. coli using standard techniques, is readily modified to construct expression vectors that produce Zyme in a variety of organisms, including, for example, E. coli, yeast of the

5

family Saccharomycetes, and Sf9 cells derived from fall armyworm ovaries of the genus <u>Spodoptera</u>, (a commonly used host for baculovirus expression systems). [Commonly used references, such as Sambrook <u>et al.</u>, <u>supra</u>, describe these techniques.]

The current literature contains techniques for constructing AV12 expression vectors and for transfecting AV12 host cells. <u>See, e.g.</u>, U.S. Patent No. 4,992,373. The current literature also contains numerous techniques for constructing 293 expression vectors and for transfecting 293 host cells.

The construction protocols utilized for 293 cells can be followed to construct analogous vectors for other cell lines, merely by substituting, if necessary, the appropriate regulatory elements using well known techniques. Promoters which may be used, for example, include the thymidine kinase promoter, the metallothionin promoter, the heat shock promoter, immunoglobulin promoter, or various viral promoters such as the mouse mammary tumor virus promoter, SV40 promoter, herpesvirus promoters, or the BK virus promoters. In addition, artificially constucted promoters, derived from "consensus" sequences or created as hybrids of other promoters may be used in the course of practicing this invention.

The DNA compounds of the present invention also include primers and probes. Nucleic acid compounds of at least 18 consecutive base pairs which encode SEQ ID NO:1 or a part thereof are included in the present invention. Probes or primers which are DNA are preferred. Most preferred probes or primers are: SEQ ID NO:3 and SEQ ID NO:4. Those in the art will recognize the techniques associated with probes and primers as being well known.

For example, all or part of SEQ ID NO:3 or SEQ ID NO:4 may be used to hybridize to the coding sequence. The full length sequence can then be generated using polymerase chain reaction (PCR) amplification, using well known techniques. The full length sequence can be subsequently subcloned into any vector of choice.

Alternatively, SEQ ID NO:3 or SEQ ID NO:4 may be radioactively labeled at the 5' end in order to screen cDNA libraries by conventional means. Furthermore, any piece of Zyme-encoding DNA which has been bound to a filter may be saturated with total mRNA transcripts, in order to reverse transcribe the mRNA transcripts which bind.

Primers and probes may be obtained by means well known in the art. For example, once pSZyme is isolated, restriction enzymes and subsequent gel separation may be used to isolate the fragment of choice.

Another embodiment of the present invention is a genomic clone of Zyme. The preferred genomic clone is the 4.0 kilobase <u>HindIII</u> fragment from a human chromosome 19 library which hybridizes to fragments of DNA which encode SEQ ID NO:1. This can be obtained via hybridization with SEQ ID NO:2, or parts thereof. For example, SEQ ID NO:3 and SEQ ID NO:4 may be radioactively labelled and used to probe a chromosome 19 library, in order to then identify and isolate the corresponding genomic DNA.

The present invention also provides an Alzheimer's diagnostic assay wherein donor human DNA is:

1) digested with the restriction enzyme <u>Taq</u> I;

2) hybridized with labelled Zyme DNA to reveal a Zyme-related band configuration; and

3) compared to the similarly-digested and hybridized band configurations of those members of the donor's family who display or displayed the symptoms of Alzheimer's disease. The preferred Alzheimer's diagnostic assay utilizes a blood sample as the source of donor human DNA.

Since the genomic DNA is provided in the present invention and a Zyme-related restriction fragment length polymorphism is identified by the disclosure of this invention, the remainder of this procedure may be accomplished according to methods known in the art. For example, U.S. Patent 4,666,828, describes these procedures. [Numerous references, such as B. Lewin, <u>Genes</u>, at page 78 (1987), review restriction fragment length polymorphism techniques and theory.]

Host cells which harbor the nucleic acids provided by the present invention are also encompassed within this invention. A preferred host cell is an oocyte. A preferred oocyte is one which has been injected with sense mRNA or DNA compounds of the present invention. A still more preferred oocyte is one which has been injected with sense mRNA or DNA compounds of the present invention in conjunction with DNA or mRNA which encodes APP. Most preferred oocytes of the present invention are those which have been injected with sense mRNA.

Other preferred host cells are those which have been transfected with a vector which comprises SEQ ID NO:2. Preferred SEQ ID NO:2-transfected host cells include include 293, AV12, yeast and <u>E. coli</u> cells. Most preferred 293 and <u>E. coli</u> host cells are 293/pRc/Zyme, <u>E. coli</u>/pSZyme .

Also preferred is a host cell which has been co-transfected with a DNA vector which comprises SEQ ID NO:2 and a DNA vector which comprises the coding sequence of APP. 293 cells, AV12 cells, yeast cells and <u>E. coli</u> cells are especially useful co-transfected host cells.

An oocyte host cell can be constructed according to the procedure described in Lubbert, <u>et al.</u>, <u>Proceedings of the National Academy of Sciences (USA)</u>, 84:4332 (1987). DNA or RNA which encodes APP (both the 695 and 751 amino acid forms) may be obtained as described in Selkoe <u>et al.</u>, <u>Proceedings of the National Academy of Sciences (USA)</u>, 85:7341 (1988). Other host cell transfection is well known in the art. Co-transfection of cells

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may be accomplished using standard techniques. See, e.g., Gorman et al., Molecular and Cellular Biology, 2:1044 (1982).

Therefore, the present invention also provides a process for constructing a host cell capable of expressing SEQ ID NO:1, said method comprising transfecting a host cell with a DNA vector that comprises a DNA sequence which encodes SEQ ID NO:1. A preferred method utilizes 293 cells as host cells. These 293 cells may be obtained from the ATCC under the accession number ATCC CRL 1573. Another preferred method utilizes AV12 cells as host cells. AV12 cells may be obtained from the ATCC under the accession number ATCC CRL 9595. Another preferred method utilizes yeast cells of the family Saccharomycetes or the bacterium E.coli as the host cells.

The preferred process utilizes an expression vector which comprises SEQ ID NO:2 in 293 cells. Especially preferred for this purpose is pRc/Zyme.

Another preferred process comprises (a) a DNA vector which comprises SEQ ID NO:2 and (b) a DNA expression vector which encodes the APP coding sequence. A most preferred process utilizes the DNA vector pRc/Zyme. Transfected host cells may be cultured under conditions well known to skilled artisans such that SEQ ID NO:1 is expressed, thus producing Zyme in the transfected host cell.

Additionally, the invention provides a process for identifying DNA homologous to a probe of the present invention, which comprises combining test nucleic acid with the probe under hybridizing conditions and identifying those test nucleic acids which hybridize. The preferred probes for use in this method are SEQ ID NO:3 and SEQ ID NO:4. Hybridization techniques are well known in the art. See. e.g., Sambrook, et al., supra.

Assays utilizing the compounds provided by the present invention are also encompassed within this invention. The assays provided determine whether a substance is a ligand for Zyme, said method comprising contacting Zyme with said substance, monitoring Zyme activity by physically detectable means, and identifying those substances which interact with or affect Zyme.

Preferred assays of the present invention incorporate a cell culture assay, a high performance liquid chromotography (HPLC) assay or a synthetic competition assay.

Preferred cell culture assays utilize oocytes, AV12, E. coli, yeast or 293 cells which co-express nucleic acids which encode Zyme and APP. Those co-expressing cell culture assays which are preferred include those which utilize 293/pRc/Zyme. A preferred assay utilizes yeast cells, and a DNA compound which encodes amino acids 587 to 606 of APP. One method of performing the yeast assay is described in Smith and Kohorn, Proceedings of the National Academy of Sciences, USA, 88:5159 (1991), using Zyme-encoding DNA and APP-encoding DNA which comprises the Met_{596}/Asp_{597} cleavage site codons.

Most preferred oocyte assays co-express mRNA. Most preferred cell culture assays utilize Western blot analysis or radiolabelled APP as the physically detectable means. A preferred HPLC assay is one wherein the substrate utilized is a full length, eukaryotically-derived APP.

The most preferred synthetic competition assay is one wherein the substance competes with the Kunitz-like domain gene product for binding to Zyme. The most preferred Zyme/Kunitz domain competition assay is one wherein APP is labelled with radioisotope.

The cell culture assays may be accomplished according to the procedures detailed by F. Ausubel, et al., Current Protocols in Molecular Biology, (1989) at pages 9.1-9.5. The HPLC assay may be performed essentially as described in Hirs and Timasheff, eds, Methods in Enzymology, Volume 91, Sections V and VI (1983). The Zyme/Kunitz-like domain binding or competition assay may be performed as described by J. Bennet and H. Yamamura, Neurotransmitter Receptor Binding, (1985) Chapter 3.

The present invention also provides a method for identifying or purifying Zyme, which comprises saturating test protein with anti-Zyme antibody, eliminating anti-Zyme antibody which fails to bind, and detecting the anti-Zyme antibody which remains bound. Antibody imaging techniques are known in the art.

The following are examples of aspects of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

Example 1

Production of Zyme in 293 cells

A lyophilized aliquot of E. coli pSZyme can be obtained from the Northern Regional Research Laboratories, Peoria, Ilinois, USA 61604, under the accession number NRRL B-18971 and used directly as the culture in the process described below. This culture has been deposited with the NRRL

Plasmid pSZyme was isolated from a culture of E. coli/pSZyme by cesium chloride purification. Plasmid pSZyme was then digested with SalI and NotI. The resulting fragment was linear. DNA ligase was used to ligate this SalI-NotI fragment and a SalI-HindIII linker into a previously linearized pRc plasmid™. (Invitrogen, catalog

7

#V750-20)

Competent E. coli cells were then transfected with the newly created pRc/Zyme vector which contained SEQ ID NO:2 and selected for those cells which contained the ampicillin resistance gene by growing on am-
5 picillin-containing medium.

After transfection of the pRc/Zyme vector into E. coli, a subsequent plasmid preparation was made in order to isolate the pRc/Zyme vector. In order to transfect 293 cells with the pRc/Zyme vector, the procedure developed by Chen and Okayama was employed. C. Chen and H. Okayama, Molecular and Cellular Biology, 7:2745 (1987). These cells were used in the cell culture assay as described in Example 2.
10 Selection on the antibiotic G418 (geneticin) was included in this step to produce stable transformants in 293 cells. The colonies which grew in the presence of G418 were then used as a source of Zyme.

Example 2

15 Cell Culture Assay

Human embryonic kidney cells (293 cells) were co-transfected with pRcZyme and an APP-encoding vector. On one occasion, a vector encoding the 695 amino acid APP (which lacks a Kunitz-like domain) was cotransfected with pRcZyme. On another occasion, a vector encoding the 751 amino acid APP (with the Kunitz-like
20 domain) was cotransfected with pRcZyme.

Transfection was achieved using standard calcium phosphate transfection. Other transfection protocols, such as described by Sambrook, et al., supra, are also effective. Amyloidogenic fragments were detected when the 695 amino acid (without KPI) APP coding sequence was used, via Western Blot analysis, as described in Sambrook, et al., supra, using antisera to the carboxy-terminal amino acids of the APP protein. Anti BX6, as
25 decribed in T Oltersdorf, et al., Journal of Biological Chemistry, 265:4492-4497 (1991), was used in this procedure. Amyloidogenic fragments were not detected when the 751 amino acid (with KPI) APP was used.

Example 3

30 HPLC Assay

Full length APP is produced in cells which have been infected with APP-encoding baculovirus. This procedure is accomplished according to J. Knops, et al., Journal of Biological Chemistry, 266:7285 (1991),. APP is then incubated in the presence of active Zyme and test compound. APP fragments are subsequently sepa-
35 rated by high performance liquid chromotography. Each pooled fragment is then microsequenced using standard, such as those of Hirs and Timasheff, eds, Methods in Enzymology, Vol. 91 Sections V and VI, (1983). The quantity of amyloidogenic fragments (those which terminate at either Met_{596} or ASP_{597}) generated are compared to the quantity generated in the absence of test compound to determine the ability of the test compound to affect Zyme.
40

Example 4

Zyme/Kunitz-like Domain Competition Assay

45 A peptide representing the KPI domain of APP is synthesized and labelled with the isotope iodine-125 (^{125}I). Competition binding assays are then conducted according to J.P. Bennet and H. Yamamura, Neurotransmitter Receptor Binding 61 (1985). Zyme is then bound to plastic microtitre wells as in the traditional ELISA assay. One such typical protocol for this step is described in F. Ausubel F., Current Protocols in Molecular Biology, 2:11.1-11.3 (1989). Radiolabelled KPI domain and unlabelled competitor compound is subsequently added
50 to the wells of the 96-well microtitre plate. The wells are then washed. The remaining isotope is recorded in order to calculate the relative affinity of the unlabelled competitor compound to Zyme.

Example 5

55 Isolating the Genomic Clone

A genomic library specific for human chromosome 19 genomic library in Charon 21A bacteriophage was purchased from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, USA 20852, (ATCC) (Catalog number 57711). These phage were transfected into E. coli K802 rec A⁻ host strain (Cat. no.

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47026). The titre of the phage was 6.5-7.0 X 10⁴ plaque forming units per microliter. A genomic clone of the gene encoding Zyme was isolated by conventional screening of phage libraries (See. e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual 2.6-2.114, 1989).

5   A radiolabelled cDNA probe was synthesized utilizing the polymerase chain reaction (such as that described by Schowalter and Sommer, Analytical Biochemistry, 177:90-94, 1989) by specifically annealing SEQ ID NO: 5 and SEQ ID NO: 6 primers to an EcoRI/NotI purified (Bio-Rad Laboratories, P.O. Box 708, Rockville Centre, New York USA, 11571, catalog number 732-6010) pRc-Zyme DNA fragment.

       Hybridization and washing was carried out at 65°C as described in the Zeta-Probe™ blotting membrane
10   instruction manual (Bio-Rad, catalog number 164-0153). Putative primary Zyme bacteriophage were stored in SM buffer containing 2-3 drops of chloroform. A single homogenous plaque (711-4) was subsequently isolated from a tertiary screen. Isolation of lambda bacteriophage DNA positive by in situ hybridization to Zyme was accomplished using standard techniques.

       Purified lambda phage Zyme DNA was digested with HindIII and electrophoresed on a 1% agarose/TBE
15   (0.1 M Tris-HCl pH 8.3, 0.1 M boric acid, 1 mM ethylenediaminetetraacetic acid) gel. Separated DNA was then transferred onto a Zeta-Probe™ blotting membrane (0.5x TBE running buffer, constant 80 volts for 1 hour) as described in section 2.5 of the Zeta-Probe™ instruction manual using non-denaturing conditions, then denatured (0.4M NaOH for 10 minutes) as described in section 2.8 of the Zeta-Probe™ instruction manual.

       A radiolabelled probe encompassing the BamHI/XbaI fragment of pRc/Zyme was used with a random
20   primed DNA labelling kit (such as that which is commercially available by Boehringer Mannheim Corporation, 9115 Hague Road, P.O. Box 50414, Indianapolis, Indiana, USA 46250-0414, catalog number 1004760) to determine if the 3' coding sequence was found in our clone. Hybridization and washing to the above Zeta-Probe™ membrane was performed as previously described and autoradiography revealed homology to the 3' region of Zyme.

25   To confirm that phage 711-4 contained the 5' Zyme coding region, the polymerase chain reaction using SEQ ID NO:7 and SEQ ID NO:8 was again utilized to specifically amplify a 470 base pair band from tertiary plaque purified chromosome 19 Zyme phage DNA according to Kainz, et al., Analytical Biochemistry, 202:46 (1992). This DNA fragment was purified, then subcloned into the pUC 19 expression plasmid, described supra. The identity of the DNA sequences corresponding to sequences 1 to 33 of the 5' Zyme cDNA coding region
30   and an additional 272 nucleotides upstream of the 5' Zyme coding region were confirmed by DNA sequence analysis, using standard techniques.

Plasmid Deposits

35   Under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for Purposes of Patent Procedures the following culture has been deposited with the permanent culture collection of the Northern Regional Research Center (NRRL), Agricultural Research Service, U.S. Department of Agriculture, 1815 N. University Street, Peoria, Illinois, 61604:

Deposited Material	Accession Number
E. coli K12/ pSZyme	NRRL B-18971

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ELI LILLY AND COMPANY

(ii) TITLE OF INVENTION: PROTEASE AND RELATED DNA COMPOUNDS

(iii)NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:
      (A) ADDRESSEE: K. G. TAPPING
      (B) STREET: ERL WOOD MANOR
      (C) CITY: WINDLESHAM
      (D) STATE: SURREY
      (E) COUNTRY: UNITED KINGDOM
      (F) ZIP: GU20 6PH

(v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Diskette
      (B) COMPUTER: Macintosh
      (C) OPERATING SYSTEM: Macintosh 7.0
      (D) SOFTWARE: Microsoft Word

SEQ ID NO:1
Met Lys Lys Leu Met Val Val Leu Ser Leu Ile Ala Ala Ala Trp Ala
              5   10   15
Glu Glu Gln Asn Lys Leu Val His Gly Gly Pro Cys Asp Lys Thr Ser
            20   25   30
His Pro Tyr Gln Ala Ala Leu Tyr Thr Ser Gly His Leu Leu Cys Gly
        35   40   45
Gly Val Leu Ile His Pro Leu Trp Val Leu Thr Ala Ala His Cys Lys
    50   55   60
Lys Pro Asn Leu Gln Val Phe Leu Gly Lys His Asn Leu Arg Gln Arg
65   70   75   80
Glu Ser Ser Gln Glu Gln Ser Ser Val Val Arg Ala Val Ile His Pro
                85   90   95
Asp Tyr Asp Ala Ala Ser His Asp Gln Asp Ile Met Leu Leu Arg Leu
            100   105   110
Ala Arg Pro Ala Lys Leu Ser Glu Leu Ile Gln Pro Leu Pro Leu Glu
        115   120   125
Arg Asp Cys Ser Ala Asn Thr Thr Ser Cys His Ile Leu Gly Trp Gly
    130   135   140
Lys Thr Ala Asp Gly Asp Phe Pro Asp Thr Ile Gln Cys Ala Tyr Ile
145   150   155   160
His Leu Val Ser Arg Glu Glu Cys Glu His Ala Tyr Pro Gly Gln Ile
                165   170   175
Thr Gln Asn Met Leu Cys Ala Gly Asp Glu Lys Tyr Gly Lys Asp Ser
                180   185   190
Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Gly Asp His Leu Arg
            195   200   205
Gly Leu Val Ser Trp Gly Asn Ile Pro Cys Gly Ser Lys Glu Lys Pro
    210   215   220
Gly Val Tyr Thr Asn Val Cys Arg Tyr Thr Asn Trp Ile Gln Lys Thr
225   230   235   240
Ile Gln Ala Lys
            244

SEQ ID NO:2

```
ATGA AGAAGCTGAT     14
GGTGGTGCTG AGTCTGATTG CTGCAGCCTG GGCAGAGGAG CAGAATAAGT TGGTGCATGG     74
CGGACCCTGC GACAAGACAT CTCACCCCTA CCAAGCTGCC CTCTACACCT CGGGCCACTT    134
GCTCTGTGGT GGGGTCCTTA TCCATCCACT GTGGGTCCTC ACAGCTGCCC ACTGCAAAAA    194
ACCGAATCTT CAGGTCTTCC TGGGGAAGCA TAACCTTCGG CAAAGGGAGA GTTCCCAGGA    254
GCAGAGTTCT GTTGTCCGGG CTGTGATCCA CCCTGACTAT GATGCCGCCA GCCATGACCA    314
GGACATCATG CTGTTGCGCC TGGCACGCCC AGCCAAACTC TCTGAACTCA TCCAGCCCCT    274
TCCCCTGGAG AGGGACTGCT CAGCCAACAC CACCAGCTGC CACATCCTGG GCTGGGGCAA    334
GACAGCAGAT GGTGATTTCC CTGACACCAT CCAGTGTGCA TACATCCACC TGGTGTCCCG    394
TGAGGAGTGT GAGCATGCCT ACCCTGGCCA GATCACCCAG AACATGTTGT GTGCTGGGGA    454
TGAGAAGTAC GGGAAGGATT CCTGCCAGGG TGATTCTGGG GGTCCGCTGG TATGTGGAGA    514
CCACCTCCGA GGCCTTGTGT CATGGGGTAA CATCCCCTGT GGATCAAAGG AGAAGCCAGG    574
AGTCTACACC AACGTCTGCA GATACACGAA CTGGATCCAA AAAACCATTC AGGCCAAG     632
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SEQ ID NO:3

ATG GCT GGC GGC ATC ATA GTC AGG G   25

SEQ ID NO:4

AAC CGA ATC TTC AGG TCT TCC TGG GG   26

SEQ ID NO:5

TCG CTC TCT CCT GGG GAC ACA GA   23

SEQ ID NO:6

CCA GGT GCT ATT CCA TGT ATG TCA TAG   27

SEQ ID NO:7

TCT GTG TCC CCA GGA GAG AGC GA   23

SEQ ID NO:8

ATA GTG AAG CTG TCT TCT CAA T   22

Claims

1.   An amino acid compound which comprises the amino acid sequence

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```
Met Lys Lys Leu Met Val Val Leu Ser Leu Ile Ala Ala Ala Trp Ala
1             5               10             15

Glu Glu Gln Asn Lys Leu Val His Gly Gly Pro Cys Asp Lys Thr Ser
        20              25              30

His Pro Tyr Gln Ala Ala Leu Tyr Thr Ser Gly His Leu Leu Cys Gly
        35              40              45

Gly Val Leu Ile His Pro Leu Trp Val Leu Thr Ala Ala His Cys Lys
    50              55              60

Lys Pro Asn Leu Gln Val Phe Leu Gly Lys His Asn Leu Arg Gln Arg
65              70              75              80

Glu Ser Ser Gln Glu Gln Ser Ser Val Val Arg Ala Val Ile His Pro
            85              90              95

Asp Tyr Asp Ala Ala Ser His Asp Gln Asp Ile Met Leu Leu Arg Leu
        100             105             110

Ala Arg Pro Ala Lys Leu Ser Glu Leu Ile Gln Pro Leu Pro Leu Glu
        115             120             125

Arg Asp Cys Ser Ala Asn Thr Thr Ser Cys His Ile Leu Gly Trp Gly
    130             135             140

Lys Thr Ala Asp Gly Asp Phe Pro Asp Thr Ile Gln Cys Ala Tyr Ile
145             150             155             160

His Leu Val Ser Arg Glu Glu Cys Glu His Ala Tyr Pro Gly Gln Ile
            165             170             175

Thr Gln Asn Met Leu Cys Ala Gly Asp Glu Lys Tyr Gly Lys Asp Ser
        180             185             190

Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Gly Asp His Leu Arg
        195             200             205

Gly Leu Val Ser Trp Gly Asn Ile Pro Cys Gly Ser Lys Glu Lys Pro
    210             215             220

Gly Val Tyr Thr Asn Val Cys Arg Tyr Thr Asn Trp Ile Gln Lys Thr
225             230             235             240

Ile Gln Ala Lys
            244
```

hereinafter defined as SEQ ID NO:1, or a functional equivalent thereof.

2. A nucleic acid compound which comprises an nucleic acid sequence which encodes for a compound of Claim 1 or a part thereof.

3. A nucleic acid compound as claimed in Claim 2 which comprises the sequence

```
ATGAAGAAGC TGATGGTGGT GCTGAGTCTG ATTGCTGCAG CCTGGGCAGA      50

GGAGCAGAAT AAGTTGGTGC ATGGCGGACC CTGCGACAAG ACATCTCACC     100

CCTACCAAGC TGCCCTCTAC ACCTCGGGCC ACTTGCTCTG TGGTGGGGTC     150

CTTATCCATC CACTGTGGGT CCTCACAGCT GCCCACTGCA AAAAACCGAA     200

TCTTCAGGTC TTCCTGGGGA AGCATAACCT TCGGCAAAGG GAGAGTTCCC     250

AGGAGCAGAG TTCTGTTGTC CGGGCTGTGA TCCACCCTGA CTATGATGCC     300

GCCAGCCATG ACCAGGACAT CATGCTGTTG CGCCTGGCAC GCCCAGCCAA     350

ACTCTCTGAA CTCATCCAGC CCCTTCCCCT GGAGAGGGAC TGCTCAGCCA     400

ACACCACCAG CTGCCACATC CTGGGCTGGG GCAAGACAGC AGATGGTGAT     450

TTCCCTGACA CCATCCAGTG TGCATACATC CACCTGGTGT CCCGTGAGGA     500

GTGTGAGCAT GCCTACCCTG GCCAGATCAC CCAGAACATG TTGTGTGCTG     550

GGGATGAGAA GTACGGGAAG GATTCCTGCC AGGGTGATTC TGGGGGTCCG     600

CTGGTATGTG GAGACCACCT CCGAGGCCTT GTGTCATGGG GTAACATCCC     650

CTGTGGATCA AAGGAGAAGC CAGGAGTCTA CACCAACGTC TGCAGATACA     700

CGAACTGGAT CCAAAAAACC ATTCAGGCCA AG                        732
```

or a functional equivalent or part thereof.

4. A nucleic acid vector which comprises the nucleic acid compound of Claim 3.

5. A DNA vector of Claim 4 which is pSZyme.

6. A host cell transfected with a nucleic acid vector of Claim 4.

7. A genomic clone of Zyme which comprises a 4.0 kilobase <u>Hind</u>III fragment from a human chromosome 19 library which hybridizes to fragments of DNA of the compound of claim 3 under conditions suitable for selective hybridization.

8. A process for diagnosing Alzheimer's disease or a propensity to develop Alzheimer's disease in a patient which comprises
   a) securing DNA from said patient;
   b) digesting said DNA with a restriction enzyme;
   c) hybridizing said digested DNA with a labeled nucleotide sequence corresponding to the compound of SEQ ID NO:2, or a part thereof; and
   d) comparing pattern of hybridization to similarly-digested and hybridized band configurations of those members of the donor's family who display or have displayed the symptoms of Alzheimer's disease.

9. An assay for determining whether a test substance is a functional ligand for a protein of SEQ ID NO:1, said method comprising
   a) contacting the protein with said test substance;
   b) monitoring the protein's activity by physically detectable means; and
   c) identifying those substances which interact with or affect the activity of the protein relative to a control which receives no test substance.

10. A method for expressing a nucleic acid sequence as claimed in Claim 2 in a transfected host cell, said method comprising culturing said transfected host cell under conditions suitable for gene expression.

European Patent
Office

EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	WO-A-9 207 068 (ATHENA NEUROSCIENCES, INC. ET AL.) * the whole document * ---	1-6,9-10	C12N15/57 C12N9/64 C12Q1/68
A	WO-A-9 203 542 (BOSTON UNIVERSITY) * the whole document * ---	1,9	
D,A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 174, no. 2, 31 January 1991, NEW YORK, US pages 790 - 796 C.R. ABRAHAM ET AL. 'A calcium-activated protease from Alzheimer's disease brain cleaves at the N-terminus of the amyloid beta-protein' * the whole document * ---	1	
A	WO-A-9 200 374 (E.I. DU PONT DE NEMOURS AND COMPANY) * the whole document * ---	1,9	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
A	WO-A-9 113 904 (CEPHALON, INC.) * the whole document * -----	1-6,9	C12N C12Q

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	11 AUGUST 1993	JULIA P.

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: CHYMOTRYPSIN-LIKE PROTEASES AND THEIR INHIBITORS

REACTION T=0   REACTION T=45 MIN   REACTION T=24 HR

m AU

COLUMN RETENTION TIM (MIN) IN)

PEAK 1: EVKMDAEFRHDSGYEVHHQ

PEAK 2: EVKMDAEF

PEAK 3: DAEF

PEAK 4: RHDSGYEVHHQ
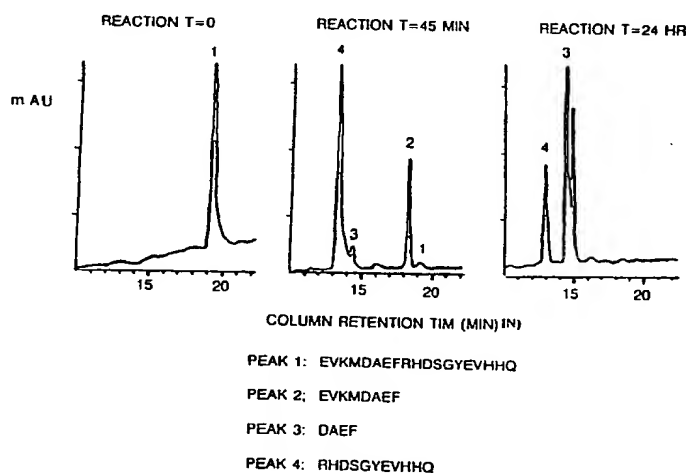
Chymase degrades an APP-mimic junction peptide between Met and Asp.

(57) Abstract

Disclosed is the identification, characterization, and purification of two chymotrypsin-like serine proteases characteristic of AD, called "chymase" and "multicatalytic protease". Both chymase and the multicatalytic protease have an enzymatic activity capable of cleaving between Met and Asp, which activity is required to generate beta-amyloid. Methods are provided for quantifying the activities of chymase and the multicatalytic protease in brain (and other tissues samples and fluids) to allow diagnosis of AD, and for development of inhibitors as therapeutic agents to slow progress of the disease. Novel compositions have been prepared and identified as inhibitors of the two enzymes. Substrates are also disclosed. Methods are provided for the treatment of AD and the identification of proteases capable of generating beta-amyloid protein. Methods are also provided for cloning and sequencing of human brain chymase and the multicatalytic protease, and the preparation of nucleic acid and antibody probes to them.

CHYMOTRYPSIN-LIKE PROTEASES AND THEIR INHIBITORS

Background of the Invention

This invention relates to chymotrypsin-like
5   proteases, and substrates and inhibitors thereof, involved
in the etiology of Alzheimer's disease.

Alzheimer's disease (AD) is a progressive
degenerative disorder of the brain that afflicts over four
million people in the United States. No effective treatment
10 is presently available. AD is only definitively diagnosed
by post-mortem histopathological methods. Even in its
end-stage, AD is sometimes confused with disorders that
manifest dementia, such as depression, which can be treated
if correctly diagnosed. Thus, there is an enormous need for
15 strategies to develop AD diagnostics and therapeutics.

An invariant feature of AD neuropathology is the
deposition of protein, known as beta-amyloid or A4, into
neuritic and cerebrovascular plaques. The presence of dense
beta-amyloid plaques in large areas of the cerebral cortex
20 is diagnostic for AD, according to guidelines of the
National Institute on Aging (Khachaturian, Arch. Neurol.
42:1097, 1985). Beta-amyloid is a 39-42 amino acid peptide
(Glenner et al., Biochem. Biophys. Res. Commun. 120:885,
1984; Masters et al., Proc. Natl. Acad. Sci. 82:4245, 1985).
25 It is synthesized as part of larger precursor proteins (Kang
et al., Nature 325:733, 1987; Tanzi et al., Nature 331:528,
1988; Ponte et al., Nature 331:525, 1988; Kitaguchi et al.,
Nature 331:530, 1988). Proteolytic processing of these
beta-amyloid precursor proteins (APP) is required to
30 generate beta-amyloid. The amino terminal of beta-amyloid
is generated by cleavage of the peptide bond in APP between

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Met[596] and Asp[597] (numbering according to Kang et al., supra).

Abraham et al. (Biotechnology 7:147, 1989) state that "it is interesting to note that the N-terminal of the
5   two cleavage sites within the precursor of the beta-protein contains a methionine and could therefore result from processing by a chymotrypsin-like protease". Similarly, Van Nostrand et al. (Nature 341:546, 1989) state that "several findings have implicated the involvement of a
10 chymotrypsin-like protease in the deposition of amyloid beta-protein in Alzheimer's disease neuritic plaques". Abraham (Neurobiol. Aging 10:463, 1989) states that "protease inhibitors are, therefore, attractive targets for drug intervention in Alzheimer's disease".
15   Pope et al. (Neurochem. Res. 9:291, 1984) describe proteases found in the human brain. They state that the neuritic plaques that characterize AD are the result of excess protein accumulation consistent with impaired steady-state proteolysis and/or protein quality control.
20 They suggest that it is likely "that comprehensive analysis of neural proteolytic machinery will be fruitful for improved understanding of the molecular and structural lesions in the cerebrospinal abiotrophies".

Among the proteases identified in mammalian brain
25 are two serine proteases that have chymotrypsin-like activity. One is an enzyme complex referred to as "ingensin" (Ishiura et al., FEBS Lett. 189:119, 1985) or the "multicatalytic protease" (Wilk et al., J. Neurochem. 40:842, 1983). This protease complex contains
30 chymotrypsin-like activity, in that it cleaves peptide bonds on the carboxy terminal side of aromatic or large aliphatic amino acids. Secondly, mast cells have been found in the

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brains of several mammals (Dropp, Acta Anat. 94:1, 1976);
these cells contain chymotrypsin-like protease (Woodbury et
al., Proc. Natl. Acad. Sci. 75:5311, 1978; Schechter et al.,
J. Immunol. 137:962, 1986).

5   There are a number of publications concerning
inhibitors for proteases and, in particular, for serine
proteases. These include: Nakajima et al., J. Biol. Chem.
254:4027, 1979; Imperiali et al., Biochemistry 25:3760,
1986; Kettner et al., J. Biol. Chem. 259:15106, 1984;

10   Kettner et al., U.S. Patents 4,582,821, 4,499,082,
4,644,055, 4,636,492, 4,652,552, and EPA 0,187,721.
        The following abbreviations are used for amino acids
and amino acid residues:

AMINO ACID	ABBREVIATION	ONE LETTER CODE
L-alanine	Ala	A
L-arginine	Arg	R
L-asparagine	Asn	N
L-aspartic acid	Asp	D
L-cysteine	Cys	C
L-glutamine	Gln	Q
L-glutamic acid	Glu	E
Glycine	Gly	G
L-histidine	His	H
L-isoleucine	Ile	I
L-leucine	Leu	L
L-lysine	Lys	K
L-methionine	Met	M
L-phenylalanine	Phe	F
L-proline	Pro	P
L-serine	Ser	S
L-threonine	Thr	T
L-tryptophan	Trp	W
L-tyrosine	Tyr	Y

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L-valine	Val	V
L-ornithine	Orn	
L-norleucine	Nle	
L-homoarginine	Harg	
L-citrulline	Cit	
L-norvaline	Nva	
L-pyroglutamic acid	Pglu	

Where prefixed by "D-", the foregoing abbreviations indicate an amino acid of D-configuration.

The following abbreviations are used for N-terminal blocking groups:

Boc	t-Butyloxycarbonyl
Ac	Acetyl
Et	Ethyl
Suc	Succinyl
MeOSuc	Methoxysuccinyl
Sub	Suberyl
Ad	Adipyl
Az	Azelayl
DNS	Dansyl
DNP	2,4-Dinitrophenyl
Z	Benzyloxycarbonyl
Fmoc	Fluorenylmethoxycarbonyl
MeOAz	Methoxyazelayl
MeOAd	Methoxyadipyl
MeOSub	Methoxysuberyl

Summary of Invention

The present invention concerns the identification, characterization, and purification of two chymotrypsin-like serine proteases characteristic of AD, called "chymase" and "multicatalytic protease", that are derived from rat or

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human brain. Both chymase and the multicatalytic protease have an enzymatic activity capable of cleaving between Met and Asp, which activity is required to generate beta-amyloid. Methods are provided for quantifying the

5 activities of chymase and the multicatalytic protease in brain (and other tissue samples and fluids) to allow diagnosis of AD, and for development of inhibitors as therapeutic agents to slow progress of the disease. Novel compositions have been prepared and identified as inhibitors

10 of the two enzymes. Methods are also provided for cloning and sequencing of human brain chymase and the multicatalytic protease, and the preparation of nucleic acid and antibody probes to them.

Thus, in a first aspect the invention features a

15 purified peptide (which term includes polypeptides and proteins) having an endopeptidase enzymatic activity which causes hydrolysis of a peptide bond between a methionine and an aspartic acid in a beta-amyloid precursor protein. By "purified" is meant that the peptide is separated from

20 components of a cell or tissue in which it naturally occurs; preferably it is purified to represent at least 70% by weight, most preferably 90%, of the peptides present in a solution or preparation.

In a second aspect, the invention features a method

25 for detection in a biological sample of an protease indicative of Alzheimer's disease. The method includes providing a substrate for the protease having the formula I:

I: R-A4-A3-A2-A1-R1

wherein R is an N-terminal blocking group or hydrogen; R1

30 is a reporter group; A4 is a covalent bond (i.e., R is directly bonded to A3), an amino acid or a peptide, e.g., including up to about five D- or L-amino acids; A3 a covalent bond (i.e., A4 is directly bonded to A2 or when A4

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is a covalent bond R is directly bonded to A2), or is a
D-amino acid, Phe Tyr, or Val or a conservative amino acid
substituent of Val, e.g., Leu, Ile, Nle, Ala, and Nva; A2 is
a hydrophobic amino acid, e.g., Leu, Ile, Nle, Phe, Val,
5   Nva, Tyr, or Pro, or preferably lysine or a conservative
amino acid substituent thereof, e.g., Arg, Orn, Cit, and
Harg or when A4 includes at least two amino acids, A2 is any
amino acid; and A1 is Met, Phe, Nle, Leu, Ile, Tyr, or
3-phenylproline; when A4 and/or A3 are a covalent bond, R
10   may be an alkane dicarboxylic acid or alkane carboxylic acid
of 2 to 20 carbon atoms, or a lower alkyl monoester thereof,
optionally substituted by an alkyl, aralkyl, or aryl
substituent; preferred substituents are isopropyl, isobutyl,
benzyl,,or phenyl, and preferred lengths are 3 to 10 carbon
15   atoms. The method also includes contacting the biological
sample with the substrate, and detecting cleavage of the
substrate as an indication of the presence of the protease
in the biological sample.

R1 is a reporter group capable of forming an amide
20   or ester linkage with the C-terminal carbonyl of amino acid
residue A1 and which, on release from the peptide sequence
by the cleavage reaction, forms a molecule R1-H or an anion
R1⁻ which may be detected by visual or instrumental means.

The precise nature of the reporter group R1 is not
25   critical to the present invention. By "reporter group" is
meant any chromogenic, magnetic, fluorogenic, luminescent,
antigenic, stable free radical or radioactive group which is
detectable by any one or more of the techniques used for
detecting particular analytes when in solution, or attached
30   to a suitable surface.

When R1 is a chromogenic group, it forms a covalent
molecule R1-H after cleavage, or a covalent ion R1⁻, which

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may optionally be separated from the unreacted peptide by
solvent extraction, precipitation, or other chemical or
physical means which allows the free R1-H or R^{1-} to be
determined visually or instrumentally. For example, when
5   R1-H is p-nitroaniline ($R1^-$ is a p-nitroanilide (pNa) ion),
it may be determined quantitatively by its ultraviolet or
visible light absorption in a photometer, or visually be
comparison with a series of standard solutions. Other
suitable chromogenic groups include nitronaphthyloxy,
10   naphthylamino, nitronaphthylamino, and quinolinamino.

When R1 is a fluorogenic group, the free $R1^-$ or
R1-H may be detected in solution by exposing the solution to
light of one wavelength and detecting fluorescence at
another, usually longer, wavelength. Suitable fluorogenic
15   groups include 4-methylcoumarin-7-amino (AMC),
2-naphthylamino, 4-trifluoromethylcoumarin-7-amino, and
4-methylumbelliferyl.

R1-H may be measured quantitatively by nuclear
magnetic resonance (NMR) by incorporating a substituent with
20   a magnetically distinctive nucleus which produces a sharp
resonance detection, and a fluoroalkyl or fluoroaryl
substituent for 19F magnetic resonance detection.

When R1 is a stable free radical (e.g., nitroxyl or
diphenylpicrylhydrazyl) substituent this permits its
25   detection by electron spin resonance (ESR).

If cleavage of R1 from the peptide is accompanied by
a marked change in the physical properties of the R1 group,
e.g., a significant change in the intensity or wavelength of
the ultraviolet absorption maximum, the physical separation
30   of the released substituent ($R1^-$ or R1-H) from the unreacted
substrate may optionally be omitted. The amount of cleavage
is determined quantitatively by measurement of the

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absorption of the released substituent R1-, at or near its
ultraviolet or visible light absorption maximum.

By "N-terminal blocking group" is meant a D-amino
acid or an arylcarbonyl, alkylcarbonyl, alkoxycarbonyl,
5   aryloxycarbonyl, aralkyloxycarbonyl, aralkylsulfonyl,
alkylsulfonyl, or arylsulfonyl peptide protecting group, or
other equivalents known to those skilled in the art of
peptide synthesis and which are known to protect molecules
from degradation by aminopeptidases (Gross and Meienhofer,
10   eds., The Peptides, vol. 3, Academic Press, New York, 1981
pp. 3-81, describes numerous suitable amine protecting
groups). As used herein, either individually or as part of
a larger group, "alkyl" means a linear, cyclic, or
branched-chain aliphatic moiety of 1 to 20 carbon atoms;
15   "aryl" means an aromatic moiety, e.g., phenyl, of 6 to 18
carbon atoms, unsubstituted or substituted with one or more
alkyl, substituted alkyl, nitro, alkoxy, or halo groups;
"substituted alkyl" means an alkyl group having a
substituent containing a heteroatom or heteroatoms such as
20   N, O or S; "halo" means Cl or Br, and "alkaryl" means an
aryl moiety of 7 to 19 carbons having an aliphatic
substituent, and, optionally, other substiuents such as one
or more alkyl, substituted alkyl, alkoxy or amino groups.
"Aralkyl" means a linear or branched chain aliphatic moiety
25   of 7 to 18 carbon atoms including an aryl group or groups.

Examples of suitable N-terminal blocking groups
include formyl, acetyl, trifluoroacetyl, benzyloxycarbonyl
(carbobenzyloxy), substituted benzyloxycarbonyl, tertiary
butyloxycarbonyl, isopropyloxycarbonyl, allyloxycarbonyl,
30   phthaloyl, benzoyl, acetoacetyl, chloroacetyl,
phenoxycarbonyl, methoxysuccinyl, succinyl, adipyl, suberyl,
2,4-dinitrophenyl, dansyl, p-methoxybenzenesulfonyl,

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p-toluenesulfonyl, methanesulfonyl, D-serine, and D-glutamic
acid.

In preferred embodiments, the biological sample is
isolated from brain, liver, heart, muscle, white blood
5 cells, mast cells or fibroblasts, serum or cerebrospinal
fluid; the protease is chymase; the protease is
multicatalytic protease.

In a third aspect, the invention features a
substrate for a protease, having the formula I described
10 above wherein R is an N-terminal blocking group or hydrogen;
R1 is a reporter group; A4 is a covalent bond (i.e., R is
directly bonded to A3), an amino acid or a peptide, e.g.,
including up to about five D- or L-amino acids; A3 a
covalent bond (i.e., A4 is directly bonded to A2 or when A4
15 is a covalent bond R is directly bonded to A2), or is a
D-amino acid, Phe Tyr, or Val or a conservative amino acid
substituent of val, e.g., Leu, Ile, Nle, Ala, and Nva; A2 is
a hydrophobic amino acid, e.g., Leu, Ile, Nle, Phe, Val,
Nva, Tyr, or Pro, or preferably lysine or a conservative
20 amino acid substituent thereof, e.g., Arg, Orn, Cit, and
Harg or when A4 includes at least two amino acids, A2 is any
amino acid; and A1 is Met, Phe, Nle, Leu, Ile, Tyr, or
3-phenylproline; when A4 and/or A3 are a covalent bond, R
may be an alkane dicarboxylic acid or alkane carboxylic acid
25 of 2 to 20 carbon atoms, or a lower alkyl monoester thereof,
optionally substituted by an alkyl, aralkyl, or aryl
substituent; preferred substituents are isopropyl, isobutyl,
benzyl, or phenyl, and preferred lengths are 3 to 10 carbon
atoms. In preferred embodiments, A3 is Val, Nle, Nva, Ile,
30 Leu, Tyr, or Phe; A2 is Lys, Arg, Orn, Cit, or Harg; A4 is
Glu or Asp; R is Boc, Fmoc, Ac, Et, Suc, MeOSuc, Az, MeOAz,
Ad, MeOAd, Sub, MeOSub, DNS, DNP, Z, or a D-amino acid; and
R2 is pNa or AMC.

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In a fourth aspect, the invention features an inhibitor of a protease, having the formula II:

II: R-A4-A3-A2-Y

where R, A4, A3, and A2 are as described above, and Y is a
5   group reactive with the active site of the protease. These
inhibitors have therapeutic utility derived from their
ability to inhibit the enzyme "chymase" or the enzyme
"multicatalytic protease" or related enzymes. These
inhibitors include peptide sequences protected at their
10   N-terminus by a protecting group, and attached at their
C-terminus by a covalent bond to a moiety Y, which interacts
strongly with functional groups located near the reactive
site of the enzyme.

The N-terminal blocking group provides metabolic
15   stability to the composition by reducing or eliminating its
ability to be degraded by endogenous aminopeptidases. The
carboxyl terminal moiety Y allows a strong chemical
interaction, mediated by covalent or noncovalent bond
formation, at the active site of the enzyme to effectively
20   inactivate the enzyme.

Y is a group which strongly interacts with
functional groups in or near the enzyme active site and is
covalently attached to the carbonyl group of amino acid A2
by an amide bond. It is provided with a lipophilic side
25   chain substituent R2, which interacts with the lipophilic
substituent recognition site of the enzyme to be inhibited,
e.g., chymase or the multicatalytic protease, and with a
functional group R3, which can react with or interact with
an adjoining serine hydroxyl group to form a
30   transition-state analog (Rich, Protease Inhibitors, ed.
Barrett and Salversen, Elsevier, 1986, p. 167.

Y may be derived from an amino acid analog Y-H in
which Y has the formula III:

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III: -HN-CH-R3
         |
         R2
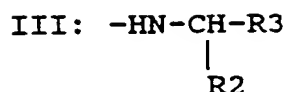
wherein R2 may be a saturated or unstaturated alkyl or
5   substituted alkyl group of 2 to 10 carbon atoms, alicyclic
or aromatic groups of 5 to 10 carbon atoms or arylalkyl
groups of up to 11 carbon atoms. Examples of suitable R2
groups include ethyl, n-propyl, isopropyl, n-butyl,
sec-butyl, isobutyl n-pentyl, n-hexyl, isohexyl, n-decyl,
10   phenyl, benzyl, beta-phenylethyl, alpha-naphthyl,
beta-naphthylmethyl, cyclohexyl, cyclohexylmethyl,
adamantyl, alpha-styryl, beta-styryl, and propargyl.

Examples of suitable R3 groups include the aldehyde
and ketone functions -CHO, $-CO-CH_3$, $-CO-CH_2Cl$, $-CO-CH_2Br$,
15   and boronic acid residues, e.g., $-B-(OH)_2$,

-B—O,   and   -B — O —(CH2)q
 | | |
 O-(CH2)$_p$ O-(CH2)$_q$-NH
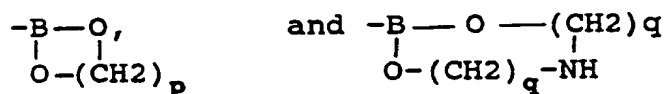
where p and q are 2 or 3; as well as alphadiketones, e.g.,
-C-C-CH3, and alphaketoesters, e.g.,   -C-C-OCH3.
 ‖ ‖   ‖ ‖
20   O O   O O
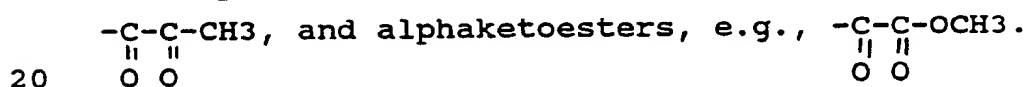
Other suitable R3 groups include $-SO_2F$, $-C_6H_4-SO_2F$,
and -PO(OR4)F where R4 is a lower alkyl or aralkyl
substituent.

Additional suitable Y groups include the acylating
25   heterocyclic ring systems described by Powers and Harper, in
Proteinase Inhibitors, A.J.Barrett and G. Salversen, eds.,
Elservier, New York, 1986, pp. 108-132. These substituents
generally interact with the enzyme active-site serine and
may subsequently react with other adjoining functionalities
30   such as histidine side chains. Examples of such Y groups
include N-substituted saccharins, as well as derivatives of
benzoxazin-4-ones, 3-alkoxy-4-chloroisocoumarins,
oxazine-2,6-diones, substituted isatoic acids,

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halomethylcoumarins, N-nitrosoisoquinolinones, haloenol
lactones, isobenzofuranones, ynenol tetrahydro-2-furanones,
2-pyranones, chloropyrones, chloroisocoumarins and the like.

5   Other Y groups may be derived from alpha-aza amino
acids and their amide, ester, and peptide derivatives of
formula IV:

$$\text{IV:} \quad \underset{\underset{R2}{|}}{H_2N-N}\overset{\overset{O}{\|}}{-C}-R5$$
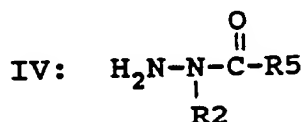
10   where R2 is as defined in formula III and R5 may be derived
respectively from an amine or ammonia, an alcohol, or a
peptide sequence substituted at its amino terminus.
Examples of the synthesis of such aza amino acids and
azapeptides are reviewed by J. Gante, Synthesis, 405-413,
15   1989. Preferred values of R5 are amino acids or peptide
sequences found at the amino terminus of beta-amyloid, e.g.,
asp, asp-ala, asp-ala-glu, asp-ala-glu-phe and their esters
and amides.

In preferred embodiments, Y has the formula:

20     $H_3N-CH-R3$,
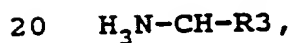
            R2

where R2 is a lipophilic side chain substituent and R3 is a
functional group which reacts with a hydroxyl group of the
protease to form a transition-state analog; R2 is a
25   saturated or unsaturated alkyl or substituted alkyl group
which includes two to ten carbon atoms or an alicyclic or
aromatic group comprising five to ten carbon atoms or an
arylalkyl group which includes eleven or fewer carbon atoms;
R2 is an ethyl, n-propyl, isopropyl, n-butyl, or sec-butyl
30   group; R3 is -CHO, -COCH$_3$, COCH$_2$Cl, or COCH$_2$Br; the protease
is chymase; the protease is mutlicatalytic protease.

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　　　　In a fifth aspect, the invention features methods
for purifying a protease to at least 70%, preferably 90% or
greater, purity from a biological sample. One method
includes homogenizing the biological sample, e.g., a

5　biological sample from a mammal such as from human, rat,
cow, or pig, in a low salt buffer including detergent;
isolating the particulate fraction from the homogenized
tissue; dissolving the particulate fraction in a high salt
buffer; separating the soluble fraction from the high salt

10　buffer; binding the protease to, and then eluting it from,
an ion exchange or a heparin column; and binding the
protease, and then eluting it from, an affinity column.

　　　　In preferred embodiments, the biological sample is
isolated from brain, liver, heart, skeletal muscle, white

15　blood cells, mast cells or fibroblasts; the low salt
includes less than 0.5M salt; the detergent is ionic or
nonionic, e.g., Triton X-100; the particulate fraction is
separated from the homogenized tissue by centrifugation,
e.g., at between 30,000 to 300,000 x g for 15 to 120

20　minutes; the high salt is greater than 0.5M salt; the
soluble fraction is separated from the high salt by
centrifugation, e.g., at 30,000 to 300,000 x g for 15 to 120
minutes; the protease is bound to a heparin column; the
protease is chymase; the protease is multicatalytic

25　protease; and the affinity column includes a substrate for
the enzyme. In this affinity cloumn the amino terminus of
the substrate is attached to the carrier column material by
a covalent bond, preferably by an amide bond; most
preferably the substrate has the formula V:

30　　　　　V:　　W-X-Y-Z
where W is deleted, an amino acid or a peptide, e.g.,
including up to about five D- or L-amino acids; X is
deleted, or is Phe, or is Val or a conservative amino acid

substituent of Val, e.g., Leu, Ile, Nle, Ala, and Nva; Y is
a hydrophobic amino acid, e.g., Leu, Ile, Nle, Phe, Val,
Nva, Pro, or Lys or a conservative amino acid substituent of
Lys, e.g., Arg, Orn, Cit, and Harg, or, when W includes at
5   least two amino acids, Y may be any amino acid; and Z is
Met, Phe, Nle, Leu, Ile, Tyr or 3-phenylproline.

Alternatively, when W is deleted, X may be a deleted
or may be an omega-aminoalkanecarboxylic acid of 2 to 20
carbon atoms in chain length, optionally substituted at the
10   alpha or beta position by an alkyl, aralkyl, or aryl
substituent. Preferred substituents are isopropyl,
isobutyl, benzyl, and phenyl, and preferred chain lengths
are 2-8 carbon atoms.

.The second method includes homogenizing the tissue
15   in a low salt buffer; isolating the soluble fraction from
the homogenized tissue; binding the protease to, and then
eluting it from, an ion exchange or heparin column;
concentrating the protease and passing it through a gel
filtration column; binding the protease, and then eluting it
20   from, an hydroxyapatite or heparin column.

In a sixth aspect, the invention features a method
for treating Alzheimer's disease. The method includes
providing an inhibitor of formula II as described above, and
administering the inhibitor to a patient in a
25   pharmaceutically acceptable carrier.

In preferred embodiments, the administration is
directly into the brain; the administration is
intramuscular, oral, or intranasal; the protease inhibitor
is an inhibitor of chymase; the protease inhibitor is an
30   inhibitor of multicatalytic protease.

In a seventh aspect, the invention features a method
for screening candidate chymase inhibitors. The method
includes the steps of: (a) incubating a chymase substrate

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(e.g., a substrate corresponding to formula I above) with one or more candidate chymase inhibitors and chymase; and (b) determining whether the one or more candidate chymase inhibitors decrease the rate of cleavage of the substrate by

5   the chymase.

In a eighth aspect, the invention features a method for screening candidate multicatalytic protease inhibitors. The method includes the steps of: (a) incubating a multicatalytic protease substrate (e.g., a substrate

10   corresponding to formula I above) with one or more candidate multicatalytic protease and multicatalytic; and (b) determining whether the one or more candidate multicatalytic protease inhibitors decrease the rate of cleavage of the substrate by the multicatalytic protease.

15   In an eighth aspect, the invention features a method for screening candidate proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp. The method includes of: (a) providing a peptide having the sequence: EVKMDAEFRHDSGYEVHHQ; (b) contacting the candidate

20   protease with the peptide; and (c) determining whether the candidate protease cleaves the substrate.

In a ninth aspect, the invention features a method for screening proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp. The method includes

25   the steps of (a) providing a substrate having the sequence R-Glu-Val-Lys-Met-R1, wherein R is hydrogen or an N-terminal blocking group, R1 is a reporter gorup; (b) contacting the candidate protease with the substrate; and (c) determining whether said candidate protease cleaves the substrate.

30   In a tenth aspect, the invention features a method for treating a patient afflicted with Alzheimer's disease. The method includes administering to the patient an

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inhibitor of chymase in a pharmaceutically acceptable
carrier.

In an eleventh aspect, the invention features a
method for treating a patient afflicted with Alzheimer's
5 disease. The method includes administering to the patient
an inhibitor of multicatalytic protease in a
pharmaceutically acceptable carrier.

By "inhibitor of chymase" is meant a molecule
capable of decreasing the ability of chymase to catalyze
10 proteolytic cleavage of a protein, particularly proteolytic
cleavage a beta-amyloid precursor protein.

By "inhibitor of multicatalytic protease" is meant a
molecule capable of decreasing the ability of chymase to
catalyze proteolytic cleavage of a protein, particularly
15 proteolytic cleavage of a beta-amyloid precursor protein.

This invention provides enzymes able to cleave a
Met-Asp bond in an APP. The quantity of such enzymes in
tissue is diagnostic of AD; their detection allows a rapid,
simple, and non-invasive assay for AD. Such detection may
20 be by enzymatic test, Western blot, or Southern or Northern
blotting techniques. Further, inhibition of the enzyme
activity *in vivo* allows progression of AD to be slowed.
Such inhibition is caused by the above described inhibitors,
and by antibodies to the enzymes.

25 Other features and advantages of the invention will
be apparent from the following description of the preferred
embodiments thereof, and from the claims.

Description of the Preferred Embodiments
The drawings will first briefly be described.

30 ### Drawings
Fig. 1 depicts an SDS-polyacrylamide gel analysis of
purified chymase;

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Fig. 2 depicts the results of the purification scheme for brain chymase;

Fig. 3 is a depiction of a comparison of the partial amino acid sequence of purified brain chymase (Brain) and

5   the partial amino acid sequence of rat mast cell protease I (RMCP I);

Fig. 4 is a set of graphs which depict the results of column chromatography used to purify chymase;

Fig. 5 is a schematic illustration of the beta/A4

10 protein and the region of beta/A4 protein used in design protease substrates;

Fig. 6 is a set of graphs which depict the degradation of substrates brain chymase (panel A) chymotrypsin (panel B), and cathepsin G (panel C);

15 Fig. 7 is a set of graphs which illustrate the results of HPLC analysis of peptides generated by chymase degradation;

Fig. 8 depicts an SDS-polyacrylamide gel analysis purified chymase;

20 Fig. 9 depicts the results of a purification scheme for chymase;

Fig. 10 depicts the results of column chromatography used to purify chymase;

Fig. 11 depicts the results of kinetic analysis of

25 degradation of substrates by rat brain chymase;

Fig. 12 is a set of graphs which depict the inhibition of protease degradation by inhibitors;

Fig. 13 depicts inhibition of various proteases by inhibitors;

30 Fig. 14 depicts the effect of inhibitors of degradation of substrates by a protease;

Fig. 15 is a graphical representation of protease activity in various parts of a Alzheimer's disease brain;

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Fig. 16 depicts the structures of a number of
protease inhibitors.

<u>Substrates and Inhibitors</u>

Substrates useful in this invention have the general
5   formula I described above. They can be used to measure the
level of chymotrypsin-like proteases characteristic of
Alzheimer's disease in body tissues and fluids. These
compositions are peptide sequences preferably protected at
their amino termini by a blocking group and attached at the
10   carboxy termini by an amide or ester bond to a reporter
group which is released when the bond is cleaved by the
protease. Such peptide compositions also include salts of
the above general formulae. Generally, the amino acid
analogs are chosen such that their identity and sequence
15   substantially corresponds to, or is identical to, those
amino acids located immediately adjacent to and upstream of
a cleavage recognition site of the enzymes chymase or the
multicatalytic protease in an APP.

By "upstream" is meant the direction which is the
20   reverse of the direction of translation, i.e., toward the
amino terminus of a peptide.

By "substantially corresponds" is meant that a
peptide sequence comprises a like number of amino acid
residues as a reference sequence, the identity and sequence
25   of which are exactly homologous, or conservatively
substituted, in relation to the reference sequence. By
"conservatively substituted" is meant that a given amino
acid residue is replaced by a biologically similar residue.
Examples of conservative substitution include substitution
30   of one alkyl or substituted alkyl residue for another such
as Nle, Met, Ile, Val, Leu, Phe or Tyr for one another, or
substitution of one polar residue for another, such as Lys

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and Arg; Glu and Asp; or Gln and Asn. "Residue" means either an amino acid or amino acid analogue.

Acceptable salts of the compounds include the salts of free peptide acids including sodium, potassium,
5 magnesium, calcium, and ammonium salts as well as acid addition salts of the free peptide bases including both inorganic and organic acids such as hydrochloric, sulfuric, phosphoric, acetic, citric, or trifluoroacetic acids.

Preferred compounds of this invention include
10 peptide derivatives of formula I in which A1 is selected from the group Phe, Met, Leu, Ile, Nle, Tyr, or 3-phenylproline; A2 is selected from the group Lys, Arg, Orn, Cit, or Harg; A3 is selected from the group Val, Leu, Ile, Nle, Phe, Nva, Pro, or is a D-amino acid, or a covalent
15 bond (i.e., A4 is bonded directly to A2 or when A4 is a covalent bond R is bonded directly to A2); A4 is Glu, Asp, D-Glu or D-Asp, or is a covalent bond (i.e., R is bonded directly to A3); R is one of the protecting groups previously described or a D-amino acid; and R1 is selected
20 from pNa and AMC. Specific examples of such compounds are shown in Figures 11 and 13.

Inhibitors have the general formula II described above. These are designed in a manner similar to the substrates but have a reactive C-terminal group as described
25 above. Specific examples of such inhibitors include those shown in Figures 12, 13 and 16.

The above compositions can be used to measure the levels of the enzymes chymase and the multicatalytic protease in body tissues and fluids and to inhibit their
30 activity. These compositions are generally peptide or peptidomimetic sequences protected at the amino terminus by a blocking group. These compounds can be prepared by the reaction of a precursor peptide derivative, for example

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R-A4-A3-A2-OH with the amino acid derivatives H-A1-R1 or H-Y
respectively.

The peptide precursors may be prepared by well-known
synthetic procedures using either the solution phase
5   techniques described in "The Peptides: Analysis, Synthesis,
Biology" Volume I (1979), eds. Gross et al. (eds.), Academic
Press, or the solid phase techniques described in Volume II
(1980) of the above series.

The peptide precursors R-A4-A3-A2-OH with protecting
10 groups in place may be prepared by the solid phase method
using the support resin described by Mergler et al.,
Tetrahedron Lett. 29:4009 (1988) and commercially available
under the name "Sasrin" from Bachem Biosciences, Inc.,
Philadelphia, PA. 19104. The peptide sequences are built up
15 on the resin by sequential treatment of the resin with amino
acid derivatives beginning with the fluorenylmethoxycarbonyl
derivative of amino acid A2 (Fmoc-A2), in the presence of a
coupling reagent such as benzotriazolyloxytris
(dimethylamino) phosphonium hexafluorophosphate (BOP),
20 followed by treatment with piperidine to remove the Fmoc
protecting group as described by Atherton et al., J. Chem.
Soc. Perkin Transactions I, 2057 (1985). The use of the BOP
reagent is described by Castro et al., Synthesis, 751-2
(1976).

25   Subsequently the Fmoc derivative of A3 and the
corresponding components of R-A4 (see below) are added with
Fmoc deblocking by piperidine between successive amino acid
additions. The functional side chains of the amino acids
A2, A3, and A4 are protected as follows: lysine is protected
30 by the t-butoxycarbonyl (Boc) group; histidine and cysteine
are protected by the N-triphenylmethyl (trityl) group;
arginine is protected with the
4-methoxy-2,3,6-trimethylphenylsulfonyl (Mtr) group;

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tyrosine, serine, and threonine are protected as the t-butyl
ethers (t-Bu); and glutamic and aspartic acids are protected
as the t-butyl esters (Ot-Bu). These particular protecting
groups remain on the peptide-resin complex throughout the
5   solid phase synthesis of peptide R-A4-A3-A2 on the resin as
well as during the cleavage of the peptide precursor
R-A4-A3-A2-OH from the resin with a dilute solution of
trifluoroacetic acid (TFA) in methylene chloride.

Alternatively, this peptide precursor may be built
10 up by solution phase methods using the strategies described
in Gross and Meienhofer, supra (volume I). The solution
phase strategy is particularly well suited to shorter
peptides in which A4 is one or two amino acids. Thus, where
A4 is a single amino acid, a peptide is built up by reacting
15 the Fmoc derivative of A4 as a N-hydroxysuccinimide ester
(Fmoc-A4-OSu) with a buffered solution of amino acid A3.
The resulting Fmoc-A4-A3-OH can next be reacted with the
benzyl ester of amino acid A2 (A2-OBzl) to give Fmoc
A4-A3-A2-OBzl. Alternatively, Fmoc-A4-OSu can be reacted
20 directly with the dipeptide ester A3-A2-OBzl to give the
same product.

Fmoc-A4-A3-A2-OBzl is next treated with piperidine
to remove the Fmoc protecting group, and can be reacted with
R-A5 (where A5 is an optional amino acid, see below) in the
25 presence of BOP to give R-A5-A4-A3-A2-OBzl. Alternatively,
Fmoc-A3-A2-OBzl can be prepared and reacted to give
R-A4-A3-A2-OBzl using this methodology.

The terminal group R-A5 (or R-A4) may be prepared by
treatment of the side-chain-protected terminal amino acid
30 group A5-OH with an acylating derivative of R. Thus when R
is an acyl (e.g., alkylcarbonyl, arylcarbonyl,
alkoxycarbonyl, aryloxycarbonyl, or aralkyloxycarbonyl) or a
sulfonyl (e.g., arylsulfonyl, alkylsulfonyl, or

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aralkylsulfonyl) function, the group R may be attached to
the side-chain-protected group A5-OH using the acid chloride
of R (R-Cl), or the symmetric anhydride of R (R-OR), or the
mixed carbonic anhydride of R (e.g., R-O-COO-isobutyl) or
5   preferably an active ester of R such as its
N-hydroxysuccinimide ester in the presence of a pH-buffering
agent such as sodium bicarbonate or N-methylmorpholine.

When the N-terminal group R is a D-amino acid it may
most conveniently be added to the side-chain-protected amino
10 acid function A5-OH as the Boc derivative of R to give
Boc-R-A5-OH. The Boc protecting group is then carried
throughout the synthesis of the protected epeptides and is
removed at the end by treatment with TFA.

Side-chain-protected Fmoc-A4-A3-A2-OBzl prepared as
15 described above is next deblocked with piperidine to give
A4-A3-A2-OBzl which is, in turn, condensed with
side-chain-protected R-A5-OH using BOP or diisopropyl
carbodiimide to yield R-A5-A4-A3-A2-OBzl. Chemical
reduction or catalytic hydrogenation of this latter compound
20 gives the side-chain-protected precursor peptide derivative.

This side-chain-protected precursor peptide is then
converted to an active ester or a mixed anhydride (Gross and
Meienhofer, I, loc. cit.) which in turn is reacted with H-Y
to yield the inhibitors of formula II.

25   The substrate compounds H-A1-R1 are purchased or are
prepared by treatment of an active ester of Boc-A1 with H-R1
followed by treatment of the resulting Boc-A1-R1 with
hydrochloric acid or TFA. Thus Boc-Phe hydroxysuccinimide
(Fluka Chemical Corp., Ronkonkoma, N.Y.) is treated with
30 p-nitroaniline to give Boc-Phe-p-nitroanilide. Treatment of
this with TFA gives Phe-p-nitroanilide.

The compounds H-Y are synthesized by methods well
documented in the literature:

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Where H-Y is an alpha amino aldehyde (i.e., R3 is
CHO), the corresponding methyl, ethyl or butyl acetal is
prepared as described by Gacek et al., Tetrahedron 30, 4233
(1974) or by Edwards, European Patent Application 291234
5   filed May 5, 1988.

Where H-Y is a chloromethyl ketone (i.e., R3 is
$COCH_2Cl$) the hydrochloride salt of the chloromethyl ketone
may be purchased (Bachem Bioscience, Inc., Philadelphia,
PA.) or can be prepared by conversion of the Boc-protected
10   amino acid to a mixed carbonic anhydride followed by
reaction with diazomethan to give diazolktones, then with
anhydrous hydrogen chloride (Kettner et al., Arch. Biochem.
Biophys. 162:56 (1974) and Fittkau, J. Prakt. Chem.
315:1037, 1973). The corresponding bromomethyl ketones
15   (i.e., R3 is $COCH_2Br$) may be prepared similarly by the
substitution of hydrogen bromide for hydrogen chloride (C.
Kettner et al., *supra*). The corresponding methyl ketones
are prepared by hydrogenolysis of the chloromethyl ketones
with palladium-on-charcoal in methanol (Kettner et al.,
20   U.S. Patent 4,652,552).

Where H-Y is a boroamino acid (i.e., R3 is $B(OH)_2$)
derivative, the cyclic ester of the boroamino acid is
prepared substantially as described by Shenvi, U.S. Patent
4,537,773 and after coupling to the precursor peptide may
25   optionally be converted to the free peptide boronic acid as
described by Shenvi and Kettner in U.S. Patent
4,499,082 and J. Biol. Chem. 259:15106 (1984).

Where H-Y is an alphadiketone or alpha-keto ester
(i.e., R3 is $COCOCH_3$ or $COCOOC_2H_5$) the requisite amino
30   diketones or alpha-keto esters may be prepared as described
by Angelastro et al., J. Med. Chem. 33:13 (1990) and
references therein.

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Where H-Y is a trifluoromethyl ketone (i.e., R3 is COCF$_3$) the requisite amino ketones may be prepared as described by Imperiali and Abeles, Biochemistry 25:3760 and supplementary pages (1986).

5   The amino-terminal substituents W and R in formulas II, III and V are derived from the corresponding carboxylic acids W-OH and R-OH which may be purchased commercially (e.g., from Narchem Corporation, Chicago, IL) or prepared as described in the following paragraphs using
10   techniques familiar to those skilled in the art.

The aminoalkanecarboxylic acids (W-OH as required for formula V compounds) may be prepared by sequential alkylation of ethyl malonate by an alkyl or arylalkyl bromide followed by an omega-phthalimido-alpha-bromoalkane.
15   The resulting substituted malonic ester may be hydrolyzed by mild base and decarboxylated by treatment with mild acid to yield an omega-phthalimido-alpha-alkyl (or arylalkyl)-substituted alkanecarboxylic acid. This intermediate may be chain extended by the Arndt Eistert homologation reaction
20   (Eistert in *"Newer Methods in Preparative Organic Chemistry"*, 1:513). Finally, strong base hydrolysis is used to remove the phthalimido protecting group to give W-OH.

The substituted alkane carboxylic acids (R-OH) as required in formulas I and II may similarly be prepared by
25   the sequential alkylation of ethyl malonate with alkyl or arylalkyl bromides follow as above by mild basic hydrolysis and mild acid decarboxylation. The resulting alpha-substituted alkane carboxylic acid may be extended as above by the Arndt-Eistert homologation reaction.

30   The omega monoesters of substituted alkanecarboxylic acids (R-OH as required in certain cases in formulas I and II) may be prepared by the sequential alkylation of ethyl

malonate with an alkyl or arylalkyl bromide followed by alkylation with the methyl or ethyl ester of an omega-bromo (or -chloro) alkane carboxylic acid. Followed by mild acid hydrolysis and decarboxylation the resulting alpha-substituted alkane dicarboxylic acid omega-ester may be extended as above by the Arndt-Eistert homologation reaction.

The resulting racemic carboxylic acids (W-OH and R-OH) may be resolved into their enantiomeric forms by passage through a chiral chromatographic column or by conversion to diastereoisomeric salts of asymmetric bases such as quinine, cinchonine, brucine and the like followed by fractional crystallization.

Example 1: Preparation of Precursor Peptides

These compounds were prepared by solution phase methods using N-hydroxysuccinimide esters (Anderson et al., J. Am. Chem. Soc. 86:1839, 1964) or mixed carbonic anhydrides (Anderson et al., J. Am. Chem. Soc. 89:5012, 1967).

The N-hydroxysuccinimide esters were prepared by treatment of a solution of 2 grams of the protected amino acid or peptide in 25 ml of dioxane with a molecular equivalent of N-hydroxysuccinimide and a molecular equivalent of diisopropylcarbodiimide (DIPCDI). The solution was stirred overnight at room temperature, and solvent was then evaporated under reduced pressure. The residue was dissolved in ethyl acetate, washed with water, 5% aqueous citric acid, and again with water. The mixture was dried ($MgSO_4$), filtered, and the solvent removed under reduced pressure on a rotary evaporator. The purity of the resulting solid product was verified by thin layer chromatography. From 2.0g of Fmoc-Val, 0.75g of

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N-hydroxysuccinimide and 0.70 ml of DIPCDI was obtained 2.6g
of Fmoc-Val-N-hydroxysuccinimide ester (Fmoc-Val-Osu).   6.5g
of succinic acid monomethyl ester and 6.1g of
N-hydroxysuccinimide and 6.5 ml of DIPCDI in 60 ml of

5   dioxane yielded 8.4g of Methoxysuccinyl N-hydroxysuccinimide
ester (MeOSuc-Osu). In the last case it was found that the
water washing caused a significant loss of product, thus
minimum quantities of water were employed.

These N-hydroxysuccinimide esters belong to a group

10   of compounds called active esters which react readily with
the primary or secondary amino groups of amino acids in
aqueous or partially aqueous solution to yield peptides in
high yield as follows:

    a)   MeOSuc-Glu(t-Bu)

15   A solution of 1.0g glutamic acid-gamma-t-butyl ester
in 5 ml water and 0.4 ml triethylamine was treated with a
solution of 1.1g MeOSuc-Osu in 10 ml dioxane. The solution
was stirred for 3 hours and the organic solvents were
removed under reduced pressure on a rotary evaporator. The

20   residue was taken up in 50 ml ethyl acetate and the
resulting solution was washed once with water, three times
with 5% aqueous citric acid, and finally with water. The
organic layer was dried ($MgSO_4$), and the solvent was removed
under reduced pressure. The residual, pale yellow oil, was

25   dissolved in ether and 400 μls dicyclohexylamine was added.
The resulting crystalline precipitate was filtered off and
washed with small portions of ether and petroleum ether.
The yield of N-Methoxysuccinyl glutamic acid gamma-t-butyl
ester (MeOSuc-Glu (t-Bu)) was 0.9 gram.

30   b)   Fmoc-Val-Lys (Boc)

A suspension of 1.0g epsilon-t-butyloxycarbonyl
lysine in 10 ml water and 0.40 ml N-methylmorpholine (NMM)

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was treated with a solution of 1.3g Fmoc-Val-Osu in 10 ml
dioxane. The initially cloudy suspension became clear after
stirring overnight. The resulting solution was treated with
20 ml 2% aqueous sodium bicarbonate solution and was twice
5   extracted with ether. The ether extracts were discarded,
and the aqueous solution acidified with dilute hydrochloric
acid to pH 1.5 and extracted with three 75 ml portions of
ethyl acetate. The combined extracts were washed once with
water and dried (MgSO4), and solvent was removed under
10   reduced pressure to yield 1.2g solid Fmoc-Val-Lys(Boc).

        c) MeOSuc-Glu(t-Bu)-Val-OBzl
        A solution of 11.6g Fmoc-Glu(t-Bu)-Val-OBzl in 50 ml
methylene chloride was treated with 20 ml piperidine and
stirred for 30 minutes at room temperature. Solvent was
15   removed under reduced pressure and remaining traces of
piperidine removed by the repeated addition and evaporation
of ethyl acetate portions. The residual
gamma-t-butyl-glutamyl valine benzyl ester was a pale yellow
solid. It was dissolved in 30 ml of a 1:1 mixture of
20   dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) and
mixed with a solution of the monomethyl succinate acid
N-hydroxysuccinimide ester obtained from 40 millimoles
(5.28g) of methoxysuccinic acid. To this solution 2.6 ml
NMM was added, and the reaction mixture stirred at room
25   temperature overnight. Ethyl acetate (250 ml) was added,
and the resulting solution extracted once with 100 ml water,
three times with 100 ml 3% citric acid, three times with 100
ml 2% sodium bicarbonate and finally with 100 ml water. The
organic layer was dried (MgSO$_4$) and evaporated to yield 7.8
30   grams of pale yellow solid N-methoxysuccinyl
gamma-t-butylglutamyl valine benzyl ester.

        d) MeOSuc-Glu(t-Bu)-Val-Osu

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A solution of 7.6g MeOSuc-Glu(t-Bu)-Val-OBzl in 60
ml acetic acetic acid was mixed with 2g 10%
palladium-on-carbon in 50% water and hydrogenated at 40
p.s.i. for 3.5 hours. The product was filtered, and solvent

5   evaporated to give 7.1g of sticky solid methoxysuccinyl
gamma-t-butyl-glutamyl-valine. This product was dissolved
in 60 ml tetrahydrofuran (THF), and 2.3g
N-hydroxysuccinimide and 2.6 ml DIPCDI added. The reaction
mixture was stirred overnight and solvent then evaporated.

10   The residue was stirred with 200 ml ethyl acetate and the
precipitated diisopropyl urea filtered off and discarded.
The filtrate was washed with 100 ml water, 2% sodium
bicarbonate (3x100ml) and 100 ml water, dried over $MgSO_4$,
and then evaporated. The resulting solid methoxysuccinyl

15   t-butyl glutamyl valine N-hydroxysuccinimide ester weighed
8.2 grams.

         e)   MeOSuc-Glu(t-Bu)-Val-Lys(Boc)
         The MeOSuc-Glu(t-Bu)-Val-Osu (8.2g) prepared above
was dissolved in 70 ml THF and added to a solution of 4.9g

20   epsilon-t-butyloxycarbonyl lysine in 60 ml water and 2.6 ml
triethylamine. After stirring overnight the reaction
mixture was diluted with 100 ml 2% sodium bicarbonate
solution and extracted with ether. The aqueous solution was
acidified with 3% citric acid and extracted with four 100 ml

25   portion of ethyl acetate. The combined organic layers were
washed with 100 ml water, dried ($MgSO_4$) and evaporated to
give 7.0 grams of hygroscopic solid
methoxysuccinyl-gamma-t-butyloxycarbonyl lysine.

         f)   Fmoc-Glu(t-Bu)-Val-OBzl

30   This compound, as well as several in the next
example, was prepared by a modification of the method of
Anderson et al., J. Am. Chem. Soc. 89:5012 (1967).

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A solution of 8.5 grams (20 millimoles)
N-fluorenylmethoxy-carbonyl-gamma-t-butylglutamic acid in 80
ml anhydrous DMF was treated with 2.2 ml of NMM and cooled
to -20°C under dry argon. A total of 2.6 ml isobutyl

5   chloroformate was then added dropwise. The solution was
stirred for 10 minutes and a precooled (-20°C) mixture of
6.25g (25 millimoles) valine benzyl ester hydrochloride and
3.3 ml NMM in 50 ml DMF was added slowly. The reaction
mixture was stirred overnight and then dissolved in 300 ml

10   ethyl acetate, and washed successively with water (1x100ml),
3% citric acid (3x100ml), dried (MgSO4) and evaporated. The
residue was dissolved in ethyl acetate and diluted with
ether to give 11.8g of fluffy white crystalline
fluorenylmethoxycarbonyl-gamma-t-butyl-glutamyl-valine

15   benzyl ester.

Example 2: Preparation of Peptide Substrates
        a) Fmoc-Val-Lys(Boc)-Met-pNa
        A solution of 1.1 grams of Fmoc-Val-Lys(Boc), 0.8
grams of BOP and 1.3 grams of 1-hydroxybenzotriazole (HOBt)

20   in 10 ml DMF was stirred for 10 minutes. A solution of 0.6g
methionine p-nitroanilide and 1.5 ml acetic acid in 5 ml DMF
and 0.3 ml NMM was then added. The reaction mixture was
stirred overnight and diluted with 75 ml ethyl acetate. The
solution was washed with water (2x30ml), 2% sodium

25   bicarbonate (3x30ml), water (1x30ml), 3% citric acid
(2x30ml), and water (1x30ml), and was dried (MgSO$_4$) and
evaporated under reduced pressure. The residue was
dissolved in ethyl acetate and ether was added to
precipitate 1.5g of a pale yellow solid

30   fluorenylmethoxycarbonyl-valyl-epsilon-t-butyloxycarbonyl-ly
syl-methionine-p-nitroanilide.
        b) Val-Lys(Boc)-Met-pNa

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A solution of 1.5g Fmoc-Val-Lys(Boc)-Met-pNa in 20
ml methylene chloride was treated with 10 ml pyrrolidine.
After 20 minutes the solvent was evaporated under reduced
pressure. Remaining traces of pyrrolidine were removed by
5   repeated dilution with ethyl acetate followed by evaporation
under reduced pressure. The resulting solid:
valyl-epsilon-t-butyloxycarbonyl-lysyl-methionine-p-nitroani
lide weighed 1.35g after washing with petroleum ether.

        c)   MeOSuc-Glu-Val-Lys-Met-pNa

10   A solution of 0.8g of MeOSuc-Glu(t-Bu)
dicyclohexylamine salt, 1.3g of Val-Lys(Boc)-Met-pNa, 200mg
of HOBt, 0.66g of BOP in 10 ml DMF was treated with 100 μl
of NMM. The reaction mixture was stirred for 3 days and
then diluted with ethyl acetate and washed with water,
15   citric acid, and sodium bicarbonate and water as above. The
organic layer was dried ($MgSO_4$) and evaporated to give a
pale yellow solid which was purified by elution through a
column prepared from 85g silica gel in chloroform. The
first fraction, which was eluted with 150 ml chloroform, was
20   discarded. The product was next eluted with 300 ml of a
solution of 2% methanol in chloroform. The resulting eluate
was evaporated to give a colorless solid (0.45g) which was
dissolved in 10 ml methylene chloride and 10 ml TFA. After
20 minutes the solvents were evaporated under reduced
25   pressure to give 270 mg of fluffy white solid
methoxysuccinyl-glutamyl-valyl-lysyl-methionine-p-nitroanili
de, which was purified by high pressure liquid
chromatography (HPLC) in water using a gradient of 10 to 80%
acetonitrile containing 0.1% TFA. The pure product weighed
30   80 milligrams.

        d)   MeOSuc-Glu-Val-Lys-Phe-pNa

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This compound and the following two were prepared by
a mixed carbonic anhydride procedure. A solution of 1.93g
(3.0 millimoles) of MeOSuc-Glu(t-Bu)-Val-Lys(Boc) in 10 ml
DMF and 0.33 ml NMM was cooled to -20°C, and 0.39 ml
5   isobutyl chloroformate was added. The reaction mixture was
stirred for 10 minutes at -20oC, and divided into three
portions for this and the following two sections.
     A 40% portion of the reaction solution containing
1.2 millimoles of the mixed isobutyl carbonic anhydride of
10   MeOSuc-Glu(t-Bu)-Val-Lys(Boc) was added under argon to a
solution of 285 mg phenylalanine-p-nitroanilide (Bachem
Biosciences, Philadelphia, PA.) in 5 ml DMF cooled to -15oC.
After 30 minutes the reaction mixture was transferred to ice
water and allowed to warm to room temperature over 2.5
15   hours. The reaction mixture was then diluted with 20 ml
ether to precipitate a solid, which was filtered off, washed
with ether and with petroleum ether, and dried. This solid
was dissolved in 10 ml of a 1:1 mixture of methylene
chloride and TFA. After 15 minutes, solvent was evaporated
20   under reduced pressure, and the residue was triturated with
ether to yield crude solid
methoxysuccinyl-glutamyl-valyl-lysyl-phenylalanine-p-nitroan
ilide. This was purified by HPLC in water containing 0.1%
TFA using a 40 minute gradient of 25 to 90% acetonitrile
25   containing 0.1% TFA.
          e) MeOSuc-Glu-Val-Lys-D-Phe-pNa
     This compound was made by the procedure given in
section (d) above, except that D-Phenylalanine-nitroanilide
was substituted for L-phenylalanine-p-nitroanilide.
30   f) MeOSuc-Glu-Val-Lys-Leu-pNa
     This compound was made by the procedure given in
section (d) above, except that leucine-p-nitroanilide was
substituted for phenylalanine-p-nitroanilide.

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The other protease substrates depicted in Fig. 11 were made by analogous methods.

Example 3: Preparation of Peptide Inhibitors

These compounds were prepared by the mixed carbonic
5   anhydride procedure described in the previous example. In general, a completely protected precursor peptide carboxylate is converted to a mixed isobutylcarbonic anhydride and allowed to react at low temperature with the free amino group of the A1 residue which may optionally have
10 its free functional groups protected as esters, ethers or acetals.

        a) MeOSuc-Glu-Val-Lys-Phenylalanine chloromethylketone [N-Methoxysuccinyl-glutamyl-valyl-lysyl-(1-chloro-4-phenyl-2
15 -keto-3-butylamide)].

        The mixed isobutyl carbonic anhydride of methoxysuccinyl-gamma-t-butyl-glutamyl-valyl-epsilon-t-butox ycarbonyl lysine was prepared by dissolving 1.93g (3.0 millimoles) of this protected precursor peptide in 10 ml DMF
20 and 0.33 ml NMM. The solution under dry argon was cooled to -20oC and 0.39 ml isobutyl chloroformate added. This reaction mixture was stirred for 10 minutes at -20°C, and divided into portions for this and the next two sections.

        A 20% portion of the reaction solution (containing
25 0.6 millimoles of the precursor peptide mixed carbonic anhydride) was withdrawn and added to a solution of 142 mg (0.5 millimoles) of phenylalanine chloromethyl ketone hydrochloride (Bachem Biosciences, Philadelphia, PA.). The resulting solution was allowed to stir for three hours at
30 room temperature, and then diluted with ether and filtered.

        The filtrate was diluted with petroleum ether to precipitate MeOSuc-Glu-(t-Bu)-Val-Lys(Boc)-phenylalanine

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chloromethyl keton which was collected by filtration. This
product was added to 10 ml of a 1:1 mixture of methylene
chloride and TFA. After 15 minutes the solvents were
evaporated under reduced pressure. The residue was
5   triturated with ether to give solid
MeOSuc-Glu-Val-Lys-Phenylalanine chloromethylketone. This
was purified by HPLC in 0.1% aqueous TFA on a C18 column
with a 40 minute gradient of 25 to 90% acetonitrile.

       b) MeOSuc-Glu-Val-Lys-Borophenylalanine pinacol
10   ester

      A 20% portion of the mixed carbonic anhydride of
MeOSuc-Glu(t-Bu)-Val-Lys(Boc) prepared in section (a) above
was treated with 142 mg borophenylalanine pinacol ester
(Shenvi, U.S. Patent 4,537,773; Shenvi and Kettner, J. Biol.
15   Chem. 259, 15106,1984), in 2 ml DMF chilled to -15°C. The
solution was stirred for 30 minutes at -15°C, then placed in
ice and allowed to warm to room temperature over 2.5 hours.
The reaction mixture was diluted with ether and filtered.
The filtrate was evaporated, and the residue dissolved in
20   chloroform and loaded on a silica gel column (10g; E. Merck
Keiselgel 100). The product was eluted with 1:1
chloroform:methanol and fractions were monitored by thin
layer chromatography (TLC).

      The eluate was evaporated and the residue dissolved
25   in 10 ml of 1:1 methylene chloride:TFA to remove the
protecting groups. After 15 minutes solvent was evaporated
and the residue triturated with ether. The resulting solid
peptide product was purified by HPLC using conditions
similar to those employed in section (a).

30   (c) MeOSuc-Glu-Val-Lys-Boronorleucine pinacol ester.

      A 20% portion of the mixed carbonic anhydride of
MeOSuc-Glu(t-Bu)-Val-Lys(Boc) prepared in section (a) above

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was treated with 83 mg boronorleucine pinacolester (prepared by the method described in Shenvi and Kettner, supra), in 2 ml DMF. The reaction, workup and purification were carried out as described for the boro-Phe analog in (b) above.

5   (d) MeOSuc-Glu-Val-Lys-Norleucinal

A solution of 2.0 millimoles mixed carbonic anhydride was prepared from 1.29g MeOSuc-Glu(t-Bu)-Val-Lys(Boc), 7 ml DMF, 0.22 ml NMM and 0.25 ml isobutyl chloroformate by the procedure described in

10   section (a) above.

The resulting solution was added to a solution of 500 mg norleucinal di-n-butyl acetal (Gacek et al. Tetrahedron 30:4233, 1974) in 3 ml DMF. The reaction was carried out and worked up as described in section (b) above,

15   and the resulting product purified by HPLC.

(e)   MeOSuc-Glu-Val-Lys-Phenylalaninal

This compound was prepared by the procedure of Example 3D except that phenylalaninal diethyl acetal was substituted for the norleucinal derivative, and a one hour

20   period of deprotection with 1:1 methylene chloride/TFA was employed. The product was purified by HPLC and the structure was verified by fast-atom bombardment mass spectroscopy (FAB-MS). Called M+1 = 620. Found M+1 = 620.

(f)   MeOSuc-Glu-Val-Lys-Aza-Phe-NH$_2$

25   Potassium cyanate (0.73g, 0.009 mole) was added in small portions over 30 minutes to a solution of 1.00g (0.0045 mole) of t-butyl 3-benzylcarbazate, and 4.5 ml of 4N hydrochloric acid in 5 ml of water and 5 ml of dioxane. The mixture was stirred overnight, and the white crystalline

30   product was filtered off and washed with aqueous 20% citric acid, water, and after vacuum drying weighed 0.81g This solid was decrystallized from ethyl acetate-hexane to give

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0.63g of N-t-Butoxycarbonyl-alpha-azaphenylalanine amide
(Boc-AzaPhe-NH₂), m.p. 131-131.5°C (Lit. m.p. 124-125°C,
Dutta et al., *J. Chem. Soc. Perkin I* (1975) 1712)

A solution of 0.6g of the above amide in 5 ml of
5 dioxane and 5 ml of 4N HCl in dioxane was allowed to stand 1
hr, and the solvent was evaporated and 20 ml of ether was
added. The precipitate (0.52g) was collected, washed with
ether and dried. A 0.28g portion of the solid in 3 ml of
DMF was treated with 1.2g of MeOSuc-Glu(t-Bu)-Val-Lys(Boc)
10 (described in Example 1(e) above) and 0.33g of BOP and 0.11g
of HOB$_T$ was added. The pH was adjusted by the dropwise
addition of NMM (ca. 0.2ml) using a droplet of the reaction
solution applied to moist pH paper to give a pH measurement
of 7.5. The solution was allowed to stand for 2 days and
15 was diluted with 75 ml of ethyl acetate, and was worked up
by the procedure in Example 1(c) above. The organic layer
was evaporated to give 0.25g of a gummy solid which was
taken up in 10 ml of 1:1 CH₂Cl₂/TFA. After 1 hr, 25 ml of
ether was added to precipitate 0.15g of MeOSuc-Glu-Val-Lys-
20 Aza-Phe-NH₂. The product was purified by HPLC and the
identity was verified by FAB-MS.

Proteases

Proteases of this invention are chymotrypsin-like
serine proteases derived from mammalian tissues. These
25 proteases share an unusual substrate specificity in being
able to cleave a Met-Asp bond in a beta-amyloid precursor
protein. This aberrant cleavage is required to liberate
beta-amyloid in Alzheimer's disease. The encompassed
proteases have an enzymatic activity, as described above,
30 which is similar to, or identical to, that activity
naturally occurring in such mammals. These proteases may
be isolated and purified, as generally described above, and

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used as purified preparations. Alternatively, the amino
acid sequence of the isolated protease, or tryptic or other
fragments thereof, can be determined by standard techniques
and the nucleic acid encoding that protease isolated. The
5   active site of the protease can be similarly determined by
fragmenting the enzyme and testing its activity, e.g., as
described below. Those fragments with the desired activity
are useful in this invention. Fragments of the enzyme, like
the intact enzyme, can also be created by standard
10   recombinant DNA technology. Antibodies, e.g., monoclonal
antibodies, to these proteases or fragments thereof are
created by standard technology.

　　　Purified protease can be used for the rational
design of organic molecules which fit into the enzyme active
15   site and inhibit its activity. The achievement of X-ray
crystallographic structure of purified protease at high
resolution will considerably aid this endeavor. Purified
enzyme can also be used for large-scale screening of
libraries of organic molecules to find compounds which
20   inhibit protease activity.

　　　Examples of isolation of two such proteases, chymase
and the multicatalytic protease, are described below. The
examples are not limiting to the invention. Those of
ordinary skill in the art can readily isolate other
25   proteases having such enzymatic activity using the protocol
provided below, or equivalent protocols. Similarly, other
proteases can be isolated by standard recombinant DNA
technology using the amino acid sequence of chymase or the
multicatalytic protease to allow reverse engineering of
30   nucleic acid encoding chymase, the multicatalytic protease,
or related enzymes.

　　　During the various protease purification steps,
protease activity of all fractions was measured using

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chromogenic or fluorogenic peptide analogs as substrates.
Other methods are available, known to those skilled in the
art. One specific example of an activity measurement is
described here. Protease activity assays were performed in
5   96 well microtiter plates, in a total volume of 0.2 mls.
Substrates were prepared as 10-20X concentrated stock
solutions in DMSO. Therefore, each reaction contained 5-10%
DMSO. Reactions were initiated by addition of
protease-containing samples to wells already containing
10 substrates, and were maintained at 37°C. Control wells
received either protease without substrate, or substrate
without protease. The release of reporter moities by
proteolytic cleavage was followed as a function of time by
either spectrophotometry (e.g., for pNa substrates, change
15 in absorbance with time at 405 nm) or fluorimetry (e.g., for
AMC substrates, change in fluorescence with time using
excitation at 390 nm and emission at 460 nm). Maximum
velocity of reactions was determined while substrate
hydrolysis increased linearly as a function of time.

20 <u>Example 4: Chymase purification</u>

Chymase (also known as mast cell protease I) was
purified to homogeneity from rat brain generally as follows.
Upon tissue homogenization and centrifugation, the enzyme
was present in the particulate fraction. Chymase was
25 enriched by removal of detergent-soluble proteins. Chymase
was then solubilized from the particulate fraction using
high salt, such as 1M $MgCl_2$ or 2M NaCl. Next, it was
precipitated by dialysis into a low ionic strength buffer,
and was collected by centrifugation. The pellet was
30 suspended in buffer and fractionated by chromatography on a
heparin-Sepharose column. Finally, chymase was purified to
homogeneity by affinity chromatography using a substrate

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linked to resin. Purification steps were monitored by
hydrolysis of the general chymotrypsin-like protease
substrate Suc-Ala-Ala-Pro-Phe-pNa (Nakajima et al., J. Biol.
Chem. 254:4027, 1979), and the APP substrate mimics
5 MeOSuc-Glu-Val-Lys-Met-pNa and MeOSuc-Glu-Val-Lys-Phe-pNa.
All procedures were performed at 4°C unless otherwise
indicated.

      a) tissue extractions

      Forebrains from 5-7 day old rat pups (17g) were
10 homogenized using a glass/Teflon motorized homogenizer in
295 mls 20 mM sodium phosphate (pH 7.5), 1 mM EDTA, and 1%
Triton X-100. After 30 minutes with occasional stirring,
this homogenate was centrifuged for 1 hr. at 40,000 x g in
an SS34 rotor. The pellets were resuspended in 40 ml 20 mM
15 sodium phosphate (pH 7.5), 1.2M MgCl2, and 0.1% Triton X-100
using a large Dounce homogenizer. To obtain a tight pellet
during the next centrifugation, the viscosity of the mixture
was reduced by two passages through a 20-gauge needle to
shear nucleic acids. After 40 minutes with occasional
20 stirring, the mixture was sonicated, then centrifuged as
above for 1 hr. and the supernatant retained. This
supernatant was then extensively dialyzed against a low
ionic strength buffer (5 mM sodium phosphate, pH 7.5). The
following day, particulates present in the dialysis bag were
25 first resuspended by end-to-end mixing, then the dialysate
was centrifuged as above for 1 hr. The resulting pellet was
dissolved in a total of 8 ml 20 mM sodium phosphate (pH
7.5), 1 mM EDTA, 0.1% Triton X-100 ("buffer A"), plus 0.4 M
NaCl and was used directly for the heparin agarose column.
30   b) heparin affinity chromatography

      The resuspension was sonicated, then applied in 1 ml
aliquots to a column (1.5 x 2 cm) of heparin-agarose
(Bio-Rad, Burlingame, CA.) equilibrated with buffer A plus

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0.4 M NaCl. The column was washed with the same buffer
until the concentration of eluting protein fell below 0.1
mg/ml. Proteins were then eluted from the column using a
step-wise gradient of increasing NaCl concentration in
5 buffer A. The protease showed an avidity for heparin, and
was eluted with 0.9-1.2 M NaCl, with an approximate
enrichment of 20-fold. The above methods for partial
purification of chymase (mast cell protease I) have been
described in the literature (e.g., Woodbury et al., Meth.
10 Enzymol 80:588, 1981 and references therein).

     c) Substrate affinity chromatography

     The concentrated, partially purified
chymase-containing sample was loaded onto a column
containing Sepharose resin to which chymase substrate
15 analog, Ala-Pro-Phe, had been covalently conjugated. The
free amino group of the substrate analog was linked to
Sepharose that had been activated by cyanogen bromide.
Chymase bound to the resin in the presence of high
concentrations of NaCl or $MgCl_2$, e.g., 1M NaCl, and was
20 eluted with 1M ammonium bicarbonate. Other methods are also
available for chymase elution, e.g., reduction of NaCl to
0.5 M in 40% ethylene glycol. Eluted fractions were
monitored for chymase activity as below.

     d) Analyses of purified chymase

25   The eluate from the final column contained a
closely-spaced doublet of ~25-26 kDa detectable by
SDS-polyacrylamide gel electrophoresis with ultrasensitive
silver staining (Nelson et al., J. Neurochem. 53:641, 1989;
Merril et al., Anal. Biochem. 110:201, 1981). An
30 illustration is provided in Fig. 1. The silver-stained
chymase doublet is shown in lane C. These polypeptides were
confirmed as proteases by enzymography. In this technique

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(Miskin et al., Anal. Biochem. 118:252, 1981; Nelson and
Siman, J. Neurochem., supra), protein samples that contain
proteases are fractionated by SDS-polyacrylamide gel
electrophoresis in which a protease substrate (e.g.,
5   gelatin) is covalently incorporated into the gel matrix.
Following electrophoresis, the SDS is removed and gels are
incubated at elevated temperature (e.g., 37°C) to activate
any proteases present. At locations within the gel
containing active protease, the substrate will be cleaved
10   from the gel matrix. The uncleaved substrate remaining
within the gel is stained by a protein-binding dye, such as
Coomassie blue or Amido black. With this technique,
proteases appear as light areas (where substrate has
been liberated from the gel matrix) against a darkly stained
15   background. Purified chymase was examined by enzymography,
shown in Figure 1 (Lane A is the heparin eluate, lane B the
purified chymase; the left lane shows molecular weight
standards, in kDa X 10-3). The two closely-spaced
polypeptides in the purified chymase preparation (lane C)
20   both exhibit protease activity (lane B). The chymase
activity exactly co-migrated with the protein detected by
silver staining.

      The purification scheme is summarized in Figure 2.
Starting from 18g rat brain, approximately 10μg homogeneous
25   chymase was obtained. The purified protease exhibited
characteristics of the chymotrypsin-like protease chymase:
(1) molecular weight of ~25-26 kDa; (2) hydrolysis of
peptide analog substrates with aromatic or large aliphatic
amino acids at their carboxyl terminus (e.g.,
30   Suc-Ala-Ala-Pro-Phe-pNa, MeOSuc-Ala-Ala-Pro-Met-pNa); (3)
irreversible inhibition by and formation of an SDS-stable
complex with alpha1-antichymotrypsin.

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Amino acid sequencing was performed on purified brain chymase to confirm the identity of the protease. This procedure was carried out by the Harvard Microchemistry Facility using a method modified from Aebersold et al.,
5   Proc. Natl. Acad. Sci. 84:6970 (1987). Chymase that had been eluted from the final substrate affinity column with ammonium bicarbonate was lyophilized. Next, ~150 pmol of chymase was digested with trypsin (enzyme:substrate of 1:20), and the resulting chymase fragments were separated by
10 narrow-bore reverse phase HPLC. Three fragments were chosen for automated N-terminal microsequencing on the basis of their yields and predicted lengths, and sequenced in a gas-phase sequenator. Figure 3 compares the partial amino acid sequences obtained from three fragments of the purified
15 protease with those published for authentic chymase (LeTrong et al., Biochemistry 26, 6988-6994, 1987). All 32 residues from the three domains precisely match the published sequence for chymase.

Example 5: Multicatalytic protease purification from human
20 brain

The multicatalytic protease identified in mammalian brain (Wilk and Orlowski, J. Neurochem. 40:842, 1983) has also been referred to in the literature as "ingensin" (Kamakura et al., J. Neursci. Res. 20:473, 1988), "lens
25 neutral endopeptidase" (Ray and Harris, Biochem. J. 248:643, 1987), "proteasome" (Tanaka et al., J. Biol. Chem. 261:15197, 1986); J. Biol. Chem. 263:16209, 1988), or "macropain" (Rivett, Arch. Biochem. Biophys. 268:1, 1989). We identified and purified from human brain an enzyme that
30 shares a number of features with the multicatalytic protease. The activity was present in the soluble fraction of a human brain homogenate, and bound to anion exchange and hydroxyapatite columns. Molecular weight of the protease

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was found to be extremely large (>600,000) by gel
filtration, a step that afforded significant purification of
the enzyme. Protease activity at each stage of the
purification was measured as described above. A specific
5   example of multicatalytic protease purification from human
brain is described below.

a) Tissue extraction and ion exchange chromatography
Human cerebral cortex (courtesy National
Neurological Research Bank, Los Angeles, CA.) was obtained
10   at autopsy and stored at -70°C until use. All procedures
were conducted at 4oC unless noted otherwise. Three samples
(35g) were pooled and homogenized in a Waring blendor in 200
mls 10 mM sodium phosphate (pH 7.4), 0.5 mM EDTA. Triton
X-100 was added to 0.5%, the homogenate was stirred for 1
15   hr., then centrifuged at 40,000 x g for 1 hr. The
supernatant, containing the multicatalytic protease, was
dialyzed against 20 mM Tris-HCl (pH 8.0),1% glycerol. The
following day, the dialysate was loaded on a column (150 ml
bed volume) of DEAE-Sepharose Fast Flow (Pharmacia,
20   Piscataway, N.J.) equilibrated in the dialysis buffer.
Unbound material was removed with ~300 mls dialysis buffer,
then bound material was eluted with a linear NaCl gradient
from 0 mM to 400 mM (1.5 liters total). Fractions of 15 ml
were collected and assayed for multicatalytic protease
25   activity (see below). Figure 4 (top) shows the elution
profile of the protease from this column. Protease activity
was detected in this and subsequent purification steps by
hydrolysis of the APP-mimic substrate
MeOSuc-Glu-Val-Lys-Met-pNa (see example 6 below for details)
30   in the presence of 0.05% SDS, which is known to stimulate
multicatalytic protease activity (Ishiura et al., supra).

b) Gel filtration

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Active fractions were pooled, brought to 30%
saturated ammonium sulfate, stirred 30 minutes, and
centrifuged at 40,000 x g for 30 minutes. The pellet was
discarded, while the supernatant was brought to 85%
5   saturation and stirred and centrifuged as before. The
pellet, containing the multicatalytic protease, was
suspended in 5 ml gel filtration running buffer (20 mM
Tris-HCl, pH 7.5, 1% glycerol) and run on a 500 ml bed of
Sephacryl S300HR (Pharmacia). The column was standardized
10   with molecular weight markers blue dextran (>2,000 kDa),
thyroglobulin (670 kDa), gamma-globulin (150 kDa) and
ovalbumin (43 kDa). The multicatalytic protease eluted from
the column with an apparent molecular weight of
approximately 700 kDa (Figure 4, middle).
15   c) Hydroxapatite chromatography
The human brain multicatalytic protease was further
purified using hydroxyapatite-Ultrogel (IBF, Columbia, MD.);
a 4 ml bed was equilibrated with 4 mM sodium phosphate (pH
7.4). Active fractions pooled from gel filtration were
20   diluted with equilibration buffer and loaded onto the column
at room temperature. Unbound proteins were washed off with
several column volumes of equilibration buffer, then bound
proteins were eluted with a linear gradient of sodium
phosphate of 4 mM to 400 mM (60 mls). The multicatalytic
25   protease eluted as illustrated in Figure 4 (bottom). This
chromatographic step split the human brain protease activity
into two peaks, as has previously been demonstrated for
hydroxyapatite purification of the multicatalytic protease
(Ishiura et al., supra).
30   Several features of the protease activity purified
as above indicate that it is the human brain homolog of the
multicatalytic protease: (1) size, ~700 kDa; (2)
co-purification; (3) chymotrypsin-like activity, hydrolyzing

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peptide analog substrates with aromatic or large aliphatic
amino acids at the carboxy terminus; (4) marked stimulation
of activity by low concentrations of SDS (Ishiura et al.,
supra); (5) inhibition by chymostatin, but not by several
5 common protease blockers (e.g.,
(phenylmethylsulfonylfluoride, leupeptin, pepstatin,
o-phenanthroline, EDTA).

Example 6: Identification of proteases important to
Alzheimer's disease.

10   Neuritic plaques are generated in the brain in
Alzheimer's disease by aberrant liberation of beta-amyloid
from its precursor proteins (APPs). The sequence of
beta-amyloid contained within APPs is shown in Figure 5.
Cleavage of the bond between Met[596] (M in the one letter
15 amino acid code) and Asp[597] (D) is required for beta-amyloid
formation, and has been proposed to occur by action of a
chymotrypsin-like protease (Abraham and Potter,
Biotechnology 7, 147-153, 1989); Van Nostrand et al., Nature
341, 546-549, 1989). Figure 5 illustrates a strategy
20 employed by the inventors for identifying chymotrypsin-like
proteases which can liberate beta-amyloid in Alzheimer's
disease. Two novel protease substrates were designed and
synthesized: MeOSuc-Glu-Val-Lys-Met-pNa and the 19 amino
acid peptide referred to as "junction peptide". Because
25 cleavage between -Glu-Val-Lys-Met and Asp- liberates
beta-amyloid, proteases cleaving these substrates on the
carboxy terminal side of Met could be involved in
Alzheimer's disease.

          Contrary to expectation, not all chymotrypsin-like
30 proteases were able to cleave MeOSuc-Glu-Val-Lys-Met-pNa
(Figure 6; EVKM using the one letter amino acid code). Both
chymase and the multicatalytic protease readily degraded

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this substrate, but the chymotrypsin-like enzymes cathepsin
G and chymotrypsin were essentially without effect. This is
particularly surprising, since both cathepsin G and
chymotrypsin readily degraded another substrate with Met at
5   the carboxyl terminus, MeOSuc-Ala-Ala-Pro-Met-pNa (e.g.,
Nakajima et al., supra). Selective cleavage of
MeOSuc-Glu-Val-Lys-Met-pNa by chymase and the multicatalytic
protease demonstrates that protease substrate specificity is
markedly influenced by amino acid residues upstream of the
10   cleavage site, and that not all chymotrypsin-like proteases
are capable of liberating beta-amyloid. The results
strongly implicate chymase and the multicatalytic protease
in the abnormal production of neuritic plaque beta-amyloid.
      Chymase was also tested for cleavage of a long
15   peptide whose sequence spans the junction of the amino
terminus of the beta-amyloid domain (residue Asp[597]). The
19 amino acid junction peptide,
Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-
Val-His-His-Gln (corresponding to APP residues 593 to 611,
20   numbering according to Kang et al., supra), was synthesized
by solid phase methods and purified by reverse phase HPLC.
The structure was confirmed by FAB-mass spectrometry and
automated microsequencing. Purified chymase was incubated
with purified junction peptide at an enzyme:substrate molar
25   ratio of 1:50 at room temperature. The reaction mixture was
fractionated by reverse phase HPLC immediately after adding
enzyme, as well as at 45 and 90 minutes and 24 and 48 hrs.
The intact junction peptide alone was run on the same HPLC
column to identify its retention time. In as little as 45
30   minutes, intact junction peptide disappeared and was
replaced by three prominent peptide fragments (Figure 7).
Each of these fragments was identified by automated

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microsequencing. Based on the sequences of the junction peptide fragments, chymase degraded the junction peptide between Met and Asp. This further implicates chymase in the aberrant liberation of beta-amyloid in Alzheimer's disease.

5   The multicatalytic protease purified from human brain also exhibited an unusual ability to cleave the APP-mimic substrate MeOSuc-Glu-Val-Lys-Met-pNa (see example 8 below), like chymase but unlike the chymotrypsin-like enzymes chymotrypsin and cathepsin G. This implicates the

10   multicatalytic protease in the aberrant liberation of beta-amyloid in Alzheimer's disease.

Example 7: Human homolog of chymase

Human brain contains a homolog of rat chymase. The homolog was identified using the same two assays of chymase

15   activity, hydrolysis of the chromogenic substrates Suc-Ala-Ala-Pro-Phe-pNa and MeOSuc-Glu-Val-Lys-Met-pNa, and enzymography, and was purified by a scheme similar to the one detailed above for the rat brain protease. The human brain chymase is similar to its rat counterpart in its

20   co-purification, its inhibition by alpha1-antichymotrypsin, its hydrolysis of peptide analog substrates of chymase, and its apparent molecular weight of about 22,000 on nonreducing enzymographic gels. Chymase was also detected in mast cells in human Alzheimer's disease brain using antibodies to

25   authentic human mast cell chymase (provided by N. Schechter, University of Pennsylvania, described in Schechter et al., supra). Specific examples of these methods are described below.

Samples obtained post-mortem from human cerebral

30   cortex (courtesy of the National Neurological Research Bank) were fractionated as described above for preparation of rat brain chymase. The human brain activity exhibited detergent-insolubility, high salt solubility, and low salt

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insolubility. Protease was further purified on a
heparin-Sepharose column in a manner similar to purification
of rat brain chymase. Protease activity was monitored by
hydrolysis of the substrate Suc-Ala-Ala-Pro-Phe-pNa as
5   described above for rat chymase. The enzyme bound to the
column in 0.4 M NaCl and was eluted by increasing the NaCl
concentration to 0.7-0.9 M.

       Next, chymase was purified using a chymase inhibitor
coupled to resin, lima bean trypsin inhibitor-agarose
10   (Sigma, St. Louis, MO.). This trypsin inhibitor binds to
and blocks human mast cell chymase (Schechter et al., J.
Biol. Chem. 258:2973, 1983). Chymase-containing fractions
from the heparin eluate were pooled, concentrated to 10 ml,
and loaded on a 1 ml bed volume of lima bean trypsin
15   inhibitor-agarose. The column was washed with Hepes buffer
containing 0.5 M NaCl (5 vol), then with water (5 vol).
Chymase was eluted with 2 mM HCl, and was immediately
neutralized with Tris-HCl (0.2 M final concentration, pH
8.0) containing 0.5 M NaCl. Figure 8 (right panel)
20   summarizes the purification of the human brain protease.
This enzyme hydrolyzed the general chymotrypsin substrate
Suc-Ala-Ala-Pro-Phe-pNa and also the specific substrate
MeOSuc-Glu-Val-Lys-Met-pNa. The enzyme activity was
inhibited by alpha1-antichymotrypsin. On enzymographic
25   gels, as shown in Figure 8, the human brain chymase
exhibited protease activity which co-migrated with rat brain
chymase (purified as described above) and authentic human
mast cell chymase (Schechter et al., 1983, supra). Figures
9 and 10 illustrate the results of the purification.

30   Mast cell chymase was also located in human brain by
immunohistochemical analysis using an antiserum to chymase
described above. In this procedure, thin sections of human
brain (obtained post-mortem, fixed in aldehydes, and

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embedded in paraffin) are probed with rabbit antibody to human mast cell chymase. Bound rabbit anti-chymase is then detected by an indirect immunoperoxidase procedure (Sternberger, in Immunocytochemistry, Cohen et al. eds.,

5   pp. 104-169, Wiley, New York, 1979); Siman et al., Neuron 3:279, 1989). Using this procedure, chymase immunoreactivity was located within the secretory granules of mast cells in both control and Alzheimer's disease cerebral cortex.

10   Example 8: Protease inhibitors

Inhibition of the proteases responsible for the aberrant liberation of beta-amyloid in Alzheimer's disease may slow the progression of the disease. Once the candidate proteases have been identified, on the basis of their

15   ability to cleave the Met^{596}-Asp^{597} bond in APP, the purified enzymes can be used to screen for protease inhibitors. Two examples of this approach are illustrated below, in the development of highly potent and selective inhibitors for the chymotrypsin-like proteases chymase and the

20   multicatalytic protease.

To develop inhibitors for the two proteases, over 40 peptide analog substrates were prepared by the methods described in examples 1 and 2 above. Additional substrates were purchased from Sigma or Bachem. These substrates

25   differ from one another by systematic, single residue substitutions; by comparing their cleavage by protease, the precise substrate preference of each protease was determined. In turn, this most-preferred sequence was used along with modifications of the carboxy terminal residue to

30   generate highly specific inhibitors. Those skilled in the art will recognize that a number of carboxy terminal modifications are available which convert a serine protease

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substrate into an inhibitor (e.g., Powers et al., In
Proteinase Inhibitors, Barrett et al. (eds) Elsevier, pp.
55-152, 1986). The inhibitors described below are not
limiting to the invention; using equivalent approachs,
5   nonpeptidic molecules can be identified from screening
collections of organic molecules, or can be rationally
designed based on knowledge of the enzyme active site
obtained from the series of peptide substrates.

          a) Chymase inhibitors
10   Cleavage of 54 peptide analog p-nitroanilide
substrates by brain chymase is depicted in Fig 11. At least
5 concentrations of each substrate were tested.
Double-reciprocal plots were used to generate kinetic
constants. In the figure, the Vmax/Km for each substrate is
15   presented compared with substrate #1,
MeOSuc-Glu-Val-Lys-Phe-pNa. Residues upstream of the
cleaved bond are denoted by their "P" position (P1 is
immediately upstream of the cleavage site, P2 is two
residues upstream, etc.).
20   Note that compounds 1-10 compare distinct P1 amino
acids. Among those examined, Phe was preferred by more than
8-fold over Nle, which in turn was preferred over Met and
Leu. Other residues were not cleavable. Similar systematic
comparisons were conducted for the P2, P3, and additional
25   upstream sites. Of particular note is the chymase
preferences at the P2 and P4 positions: a positively charged
side chain is preferred at P2, while a negatively charged
one is preferred at P4. This substrate specificity
distinguishes chymase from other members of the chymotrypsin
30   family. As already mentioned in example 6 above, neither
chymotrypsin nor cathepsin G cleaved a substrate with
negative charge at P4 (Glu) and positive charge at P2 (Lys),
such as MeOSuc-Glu-Val-Lys-Met-pNa.

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Based on the above chymase preferences, highly
potent and selective inhibitors of the protease were
prepared. By conversion of the carboxy terminal amino acid
in a preferred substrate to an amino aldehyde, boronic acid,
5   or chloromethylketone. Fig. 12 shows that the aldehyde,
boronic acid, and chloromethylketone analogs of
MeOSuc-Glu-Val-Lys-Phe-pNa each inhibited chymase with a Ki
of less than 20 nM. These are substantially more potent
than any chymase inhibitor previously described in the.
10 literature. Moreover, they are highly selective for
chymase, as they inhibited other chymotrypsin-like proteases
(Figure 12) or other proteases (Figure 11, panel A) only at
much higher doses. For example, MeOSuc-Glu-Val-Lys-Phe-al,
inhibits chymase with a Ki of 15 nM, but did not inhibit
15 chymotrypsin or cathepsin G at doses up to 100 times greater
(Figure 12). Furthermore, the inhibitor caused substantial
blockade of the proteases trypsin, thrombin, elastase,
urokinase, plasmin, or plasma kallikrein only at 10 uM
concentrations or higher (Figure 11, panel A).
20   The aldehyde analogs of MeOSuc-Glu-Val-Lys-Phe and
MeOSuc-Glu-Val-Lys-Nle were compared as chymase inhibitors.
The Phe compound was approximately 20 times more potent than
the Nle inhibitor, a finding consistent with the
differential cleavage of the two p-nitroanilide substrate
25 versions of these compounds.
        b) Multicatalytic protease inhibitors
        A strategy equivalent to that used to design chymase
inhibitors was employed for the human brain multicatalytic
protease. Figure 14 shows the relative hydrolysis rates of
30 48 peptide analog substrates, compared at a single substrate
concentration. Note that substrate preferences differ from
those of brain chymase. For example, while chymase prefers
Phe at P1 and Lys at P2, the multicatalytic protease prefers

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Leu at P1 (although Phe, Nle, and Met are also excellent
here) and Arg at P2. Among the residues tested thus far at
the P3 position, Ile is the most-preferred. Thus, peptide
analogs can be carefully tailored which target the
5   multicatalytic protease but spare related chymotrypsin-like
enzymes.

As illustrated above for chymase, preferred
substrates of the multicatalytic protease can be modified at
their carboxy termini to generate potent enzyme inhibitors.
10   Figure 13, panel B depicts the inhibition of the
multicatalytic protease by some peptide analogs and
commercially available protease inhibitors. The novel
peptide analog inhibitors are substantially more potent
blockers of the multicatalytic protease chymotrypsin
15   activity than any previously described inhibitor.

Example 9: AD and protease activity

Chymotrypsin-like protease activity was quantified
in a number of human brain samples, in order to determine if
chymase might be responsible for the aberrant proteolysis in
20   brain in AD. Ninety-five brain samples from four brain
regions were obtained from the National Neurological
Research Bank, Los Angeles, CA. Forty-seven were from
patients diagnosed as having AD (diagnosis confirmed at time
of death), and forty-eight were from non-demented
25   age-matched individuals who died of various causes. Chymase
was enriched and separated from other protease activities by
its insolubility in detergent and low-salt buffer, followed
by solubilization in 1 M $MgCl_2$ (high salt extract
preparation, as previously detailed). Chymase activity was
30   assessed by hydrolysis of the general chymotrypsin substrate
MeOSuc-Ala-Ala-Pro-Phe-pNa and was normalized to total
protein content.

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In AD parietal cortex, chymase activity is significantly elevated by about 2-fold (n= 17; p<0.05; Figure 15). Activity is also elevated in frontal cortex (n=24), but is unchanged in occipital cortex (n=26) and
5   cerebellum (n=28). The differences in parietal and frontal cortex could not simply be ascribed to changes as a function of age or post-mortem sample autolysis time. Moreover, activity of two other proteases, cathepsin B and prolylendopeptidase, were not elevated in the same parietal
10 cortex samples, indicating that there is no generalized increase in protease activities in AD. It is noteworthy that the two brain areas exhibiting elevated chymotrypsin-like activity are severely afflicted by AD, whereas the two areas exhibiting no elevation of protease activity are relatively
15 spared by the disease (Brun et al., Histopathology 5 549, 1981); Kemper, in K. Nandy, ed., Senile Dementia: A Biomedical Approach, Elsevier, pp. 105-113, 1978). These results provides direct support for the idea that aberrant proteolytic activity of chymase might underlie the
20 pathogenesis of AD.
Therapy
        The above inhibitors can be provided in any standard pharmaceutically acceptable buffer for administration to a patient suffering from AD or Down's Syndrome. Such
25 inhibitors can be administered by standard procedures preferably at a level between 10-1000 μg/kg, intraveneously, intramuscularly, intranasally, orally or directly into brain tissue.

Other Embodiments
30   a) Nucleic acid encoding protease
        Purified chymase or multicatalytic protease can be used for the cloning and sequencing of chymase and

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multicatalytic protease cDNA and protein, by methods now
familiar to those skilled in the art of molecular biology of
proteins. Partial amino acid sequence can be obtained from
purified human brain chymase using automated or manual

5   peptide sequencing employing Edman degradation or other
chemistries. The partial protein sequence can be used in
two ways. In one, antibodies are raised against chymase. A
synthetic peptide is prepared based on the partial sequence,
and is conjugated to a carrier protein and used to raise

10   polyclonal and monoclonal antibodies by conventional methods
(Harlow et al., Antibodies, A Laboratory Manual, Cold Spring
Harbor Laboratory, New York, 1988). In the second, chymase
cDNA is cloned and sequenced, thus providing the full
nucleotide and amino acid sequences of the protein. An

15   oligonucleotide is prepared based on the partial protein
sequence, and is used to screen a cDNA library to select
recombinant bacteria expressing chymase cDNA. Upon
selection, the chymase cDNA is cloned and sequenced by
conventional methods (Sambrook et al., Molecular Cloning, A

20   Laboratory Manual, Cold Spring Harbor Laboratory, New York,
1989).

Alternatively, oligonucleotides based on the
published amino acid sequence of rat chymase (LeTrong et
al., Biochemistry 26:6988, 1987) can be used as primers for

25   a polymerase chain reaction in the cloning of cDNA for rat
or human chymase. Either the human cDNA can be directly
obtained by using human mRNA to drive the reaction, or the
rat cDNA can be obtained (by using rat mRNA to drive the
reaction) and subsequently used as a probe for cloning the

30   human counterpart by conventional methods.

Nucleic acid probes specific for chymase or the
multicatalytic protease have utility in diagnostic formats
using readily accessible cells. Mutations, duplication, or

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overexpression of the genes for clipsin or the
multicatalytic protease can be detected by a variety of
methods known to those skilled in the art.

b) Antibodies to protease

5   Antibodies to chymase, the multicatalytic protease,
or related proteases can be used in a variety of formats
designed to detect protease aberrations which may be present
in Alzheimer's disease. These have utility as diagnostic
tests for the disease. For example, changes in the amount
10   of chymase protein, or mutations in the chymase protein
which lead to the production of altered forms, could be
detectable in readily accessible cells such as skin
fibroblasts or mast cells, or white blood cells.
Antibody-based detection could be accomplished by a number
15   of means presently in wide use by those skilled in the art,
including Western blotting and enzyme-linked immunosorbant
assay (ELISA; Harlow and Lane, Antibodies, A Laboratory
Manual, Cold Spring Harbor Laboratory, New York, 1988).

c) Protease inhibitors

20   In addition to the inhibitors described in the
examples, one skilled in the art may prepateother inhibitors
of the enzymes of the invention using the above-described
methods. A number of useful inhibitors are illustrated in
Figure 16. The delineation of the enzymatic recognition
25   requirements with the peptide substrates of the invention
thus makes possible the design of inhibitors using similarly
arranged peptide side chains. The removal of peptide bonds
from the resulting molecules is, however, a desirable goal
because the presence of such bonds may leave the inhibitor
30   accessible to the attack of proteolytic enzymes.
Additionally, such peptide bonds may affect the biological
availability of the resulting therapeutic molecules.
Several examples of the application of "depeptidization" to

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the P3 and P4 positions (nomenclature of Berger and
Schechter, Phil. Trans. R. Soc. London, Ser. B (1970)
257:249) are given in the list of equivalents in Fig. 16.
In principle, this approach may be employed at the P1 and P2
5 positions by the substitution of the amide bond -CONH- with
the spatially similar $-CH_2CH_2-$, $-CH_2-O-$, $-CH=CH-$ and $-CH_2S-$
groups.

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Claims

1. A purified peptide having an endopeptidase enzymatic activity which causes hydrolysis of a peptide bond between a methionine and an aspartic acid in a beta-amyloid
5 precursor protein.

2. A method for detection in a biological sample of a protease indicative of Alzheimer's disease, comprising the steps of:

(a) providing a substrate for said protease having
10 the formula R-A4-A3-A2-A1-R1, wherein:

R is hydrogen or a N-terminal blocking group;

A4 is a covalent bond, an amino acid or a peptide;

R1 is a reporter group;

15 A3 is a covalent bond, a D-amino acid, Phe, Tyr, Val, or a conservative amino acid substituent of Val;

A2 is a hydrophobic amino acid, or Lys or conservative amino acid substituent of Lys, when A4 includes at least two amino acids, A2 is any amino acid; and

20 A1 is chosen from the group consisting of Met, Phe, Nle, Leu, Ile, Tyr, and 3-phenylproline;

(b) contacting said biological sample with said substrate; and

(c) detecting cleavage of said substrate as an
25 indication of the presence of said protease in said biological sample.

3. The method of claim 2 wherein said biological sample is isolated from brain, liver, heart, muscle, white blood cells, mast cells, fibroblasts, serum, or
30 cerebrospinal fluid.

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4. The method of claim 2 wherein said protease is chymase.

5. The method of claim 2 wherein said protease is multicatalytic protease.

5   6. A substrate for a protease, having the formula:
R-A4-A3-A2-A1-R1, wherein
        R is hydrogen or a N-terminal blocking group;
        A4 is a covlent bond, an amino acid or a peptide;
        R1 is a reporter group;
10   A3 is a covalent bond, a D-amino acid, Phe, Tyr,
Val, or a conservative amino acid substituent of Val;
        A2 is a hydrophobic amino acid, or Lys or a
conservative amino acid substituent of Lys or, when A4
includes at least two amino acids, A2 is any amino acid; and
15   A1 is chosen from the group consisting of Met, Phe,
Nle, Leu, Ile, Tyr, and 3-phenylproline.

7. The substrate of claim 6, wherein A3 is chosen from the group consisting of Val, Nle, Nva, Ile, Leu, Tyr and Phe.

20   8. The substrate of claim 6, wherein A2 is chosen from the group consisting of Lys, Arg, Orn, Cit and Harg.

9. The substrate of claim 6, wherein A4 is chosen from Glu and Asp.

10. The substrate of claim 6, wherein R is chosen
25   from Boc, Fmoc, Ac, Et, Suc, MeOSuc, Az, MeOAz, Ad, MeOAd,
Sub, MeOSub, DNS, DNP, Z, or a D-amino acid.

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11.   The substrate of claim 6, wherein R2 is chosen from pNa or AMC.

12.   An inhibitor of a protease, having the formula R-A4-A3-A2-Y, wherein

R is hydrogen, or a N-terminal blocking group;
A4 is a covalent bond, an amino acid or a peptide;
A3 is a covalent bond, a D-amino acid, Phe, Tyr, Val, or a conservative amino acid substituent of Val;
A2 is a hydrophobic amino acid or lysine or a conservative amino acid substituent thereof or, when A4 includes at least two amino acids, A2 is any amino acid; and
Y is a group reactive with the active site of said protease.

13.   The inhibitor of claim 12, wherein Y has the formula -HN-CH-R3, wherein
$$\underset{\displaystyle R2}{|}$$

R2 is a lipophilic side chain substituent; and
R3 is a functional group which reacts with a hydroxyl group of said protease to form a transition-state analog.

14.   The inhibitor of claim 13 wherein said R2 is a saturated or unsaturated alkyl or substituted alkyl group comprising two to ten carbon atoms or a alicyclic or aromatic group comprising five to ten carbon atoms or a arylalkyl group comprising eleven or fewer carbon atoms.

15.   The inhibitor of claim 14 wherein said R2 is an ethyl, n-propyl, isopropyl, n-butyl, or sec-butyl group.

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16.   The inhibitor of claim 13 wherein said R3 group is -CHO, -COCH$_3$, COCH$_2$Cl, or COCH$_2$Br.

17.   The inhibitor of claim 12 wherein said protease is chymase.

5   18.   The inhibitor of claim 12 wherein said protease is multicatalytic protease.

19.   A method for purifying a protease to at least 80% purity from a biological sample, comprising the steps of:

10   (a) homogenizing said biological sample in a low salt buffer including detergent;

(b) isolating the particulate fraction from said homogenized biological sample;

(c) dissolving said particulate fraction in a high

15   salt buffer;

(d) isolating the soluble portion of said dissolved particulate fraction;

(e) binding said protease to an ion exchange or a heparin column and then eluting said protease from said ion

20   exchange or heparin column; and

(f) binding said protease to a substrate affinity column and then eluting said protease from said substrate affinity column.

20.   The method of claim 19, wherein said biological

25   sample is isolated from a mammal.

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21. The method of claim 20, wherein said mammal is chosen from the group consisting of human, rat, cow, and pig.

22. The method of claim 19, wherein said biological
5   sample is isolated from brain, liver, heart, muscle, white blood cells, mast cells, or fibroblasts.

23. The method of claim 19, wherein said low salt comprises less than 0.5 M salt.

24. The method of claim 23, wherein said detergent
10 is ionic or non-ionic.

25. The method of claim 24, wherein said detergent is Triton X-100.

26. The method of claim 19, wherein said particulate fraction is separated from the homogenized
15 biological sample by centrifugation.

27. The method of claim 26, wherein said centrifugation is between 30,000 to 300,000 x g for 15 minutes to 120 minutes.

28. The method of claim 27, wherein said high salt
20 is greater than 0.5 M salt.

29. The method of claim 19, wherein said soluble fraction is separated from the high salt by centrifugation.

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30. The method of claim 29, wherein said centrifugation is at 30,000 to 300,000 x g for 15 minutes to 120 minutes.

5   31. The method of claim 19, wherein said protease is bound to a heparin column.

32. The method of claim 19, wherein said affinity column comprises a substrate for said enzyme.

33. The method of claim 19, wherein said substrate has the formula: W-X-Y-Z, wherein

10   W is a covalent bond, an amino acid or a peptide;
          X is valine or a conservative amino acid substituent thereof;
          Y is a hydrophobic amino acid or Lys or a conservative substituent of Lys or, when W includes at least

15   two amino acids, Y is any amino acid; and
          Z is chosen from the group consisting of Met, Phe, Nle, Ile, Leu, Tyr, and 4-phenylproline.

34. The method of claim 19 wherein said protease is chymase.

20   35. The method of claim 19 wherein said protease is multicatalytic protease.

36. A method for treating a patient afflicted with Alzheimer's disease, said method comprising administering to said patient a protease inhibitor having the formula:

25   R-A4-A3-A2-Y, wherein
          R is hydrogen or an N-terminal blocking group;
          A4 is a covalent bond, an amino acid or a peptide;

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A3 is a covalent bond, a D-amino acid, Phe, Tyr, Val, or a conservative amino acid substituent of Val;

A2 is a hydrophobic amino acid or Lys or a conservative amino acid substituent thereof or, when A4 includes at least two amino acids, A2 is any amino acid; and

Y is a group reactive with the active site of said protease.

37. The method of claim 36, wherein said administration is directly into the brain.

38. The method of claim 36, wherein said administration is intramuscular, oral, or intranasal.

39. A method for screening candidate chymase inhibitors comprising the steps of:

(a) incubating a chymase substrate with one or more said candidate chymase inhibitor and chymase; and

(b) determining whether said one or more candidate chymase inhibitors decrease the rate of cleavage of said substrate by said chymase.

40. A method for screening candidate multicatalytic protease inhibitors comprising the steps of:

(a) incubating a multicatalytic protease substrate with one or more said candidate multicatalytic protease inhibitors and multicatalytic protease; and

(b) determining whether said one or more candidate multicatalytic protease inhibitors decrease the rate of cleavage of said substrate by said multicatalytic protease.

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41. A method for screening candidate proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp comprising the steps of

(a) providing a peptide having the sequence:
5 EVKMDAEFRHDSGYEVHHQ;

(b) contacting said candidate protease with said peptide; and

(c) determining whether said candidate protease cleaves said substrate.

10 42. A method for screening candidate proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp comprising the steps of

(a) providing a substrate having the sequence: R-Glu-Val-Lys-Met-R1, wherein
15 R is hydrogen or a N-terminal blocking group;

R1 is a reporter group;

(b) contacting said candidate protease with said substrate; and

(c) determining whether said candidate protease
20 cleaves said substrate.

43. A method for treating a patient afflicted with Alzheimer's disease, said method comprising administering to said patient an inhibitor of chymase in a pharmaceutically acceptable carrier.

25 44. A method for treating a patient afflicted with Alzheimer's disease, said method comprising administering to said patient an inhibitor of multicatalytic protease in a pharmaceutically acceptable carrier.
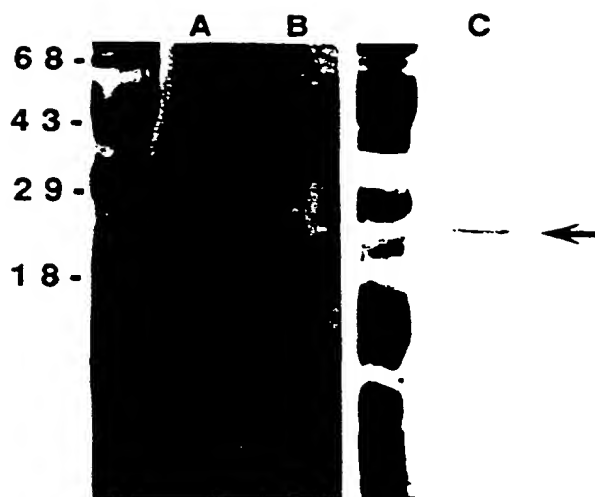
FIG. 1

FIG. 2

Purification of chymase from 7-day old rat brain

Step	Volume	Protein	Units	Specific activity	Yield	Purification
	ml	mg	nmol / sec[a]	units / mg	%	-fold
Homogenate	304	979	7,004	7	100	1
Detergent-extracted Pellet	40	180	5,043	28	72	4
Magnesium Extract	32	136	3,022	22	43	3
Low-Salt Precipitate	9	54	4,202	78	60	11
Heparin—agarose	8	1.8	2,677	1,487	38	212
Ala-Pro-Phe—Sepharose	2	0.01[b]	2,387	238,700	34	34,100

[a]One unit of activity is defined as the release of one nanomole of p-nitroaniline per sec from AAPF-pNA at pH 7.5 at 37°C.

[b]Estimated from amino acid analysis of the purified protein.

FIG. 3

Comparison between partial amino acid sequence of purified brain protease and rat mast cell protease I (RMCP I) sequence.

Brain   I I G G V E S R P H S R P [Y] = A – L E I

RMCP I   1   I I G G V E S R P H S R P Y M A H L E I   21

Brain   Q I V H P N Y

RMCP I   76   Q I V H P N Y   82

Brain   I S P Y V P [W]

RMCP I   211   I S P Y V P W   219

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Purification of human brain protease
homolog of the mulicatalytic protease

DEAE-SPEHAROSE FAST FLOW

FIG. 4a

GEL FILTRATION:
SEPHACRYL S300HR

FIG. 4b

HYDROXYAPATITE-ULTROGEL
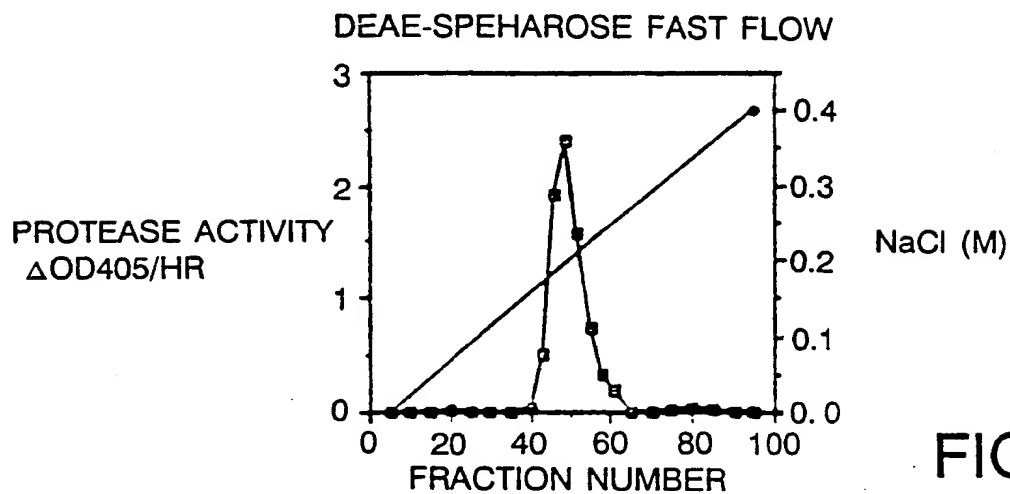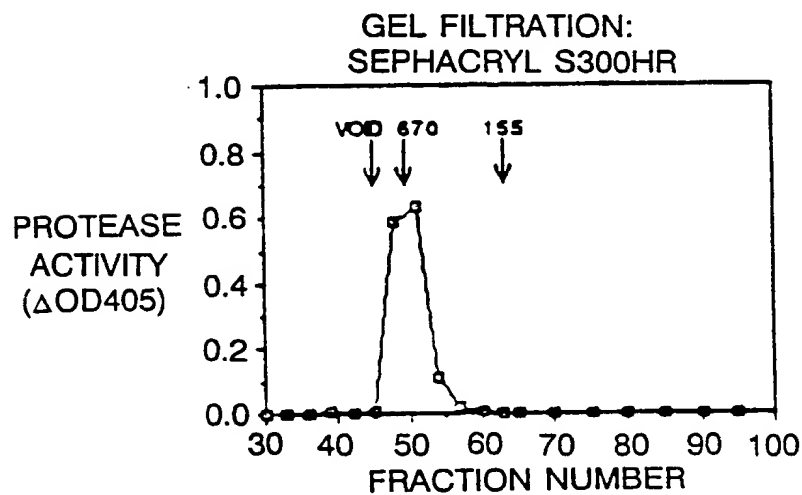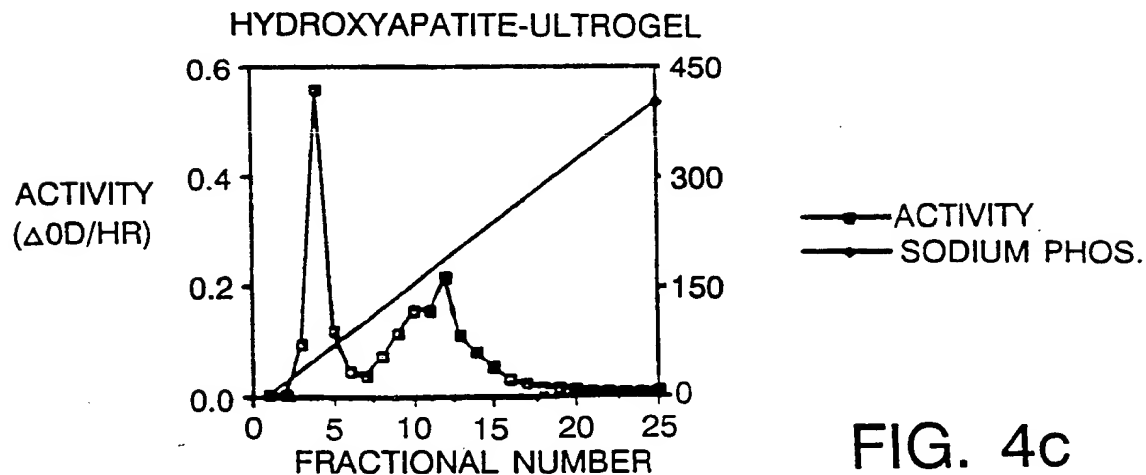
FIG. 4c

DESIGN OF PROTEASE SUBSTRATES FOR
IDENTIFICATION OF BRAIN PROTEASES
WHICH MAY GENERATE BETA/A4 PROTEIN

··· E V K M D A E F R H D S G Y E V H H Q ⁻
BETA/A4 PROTEIN
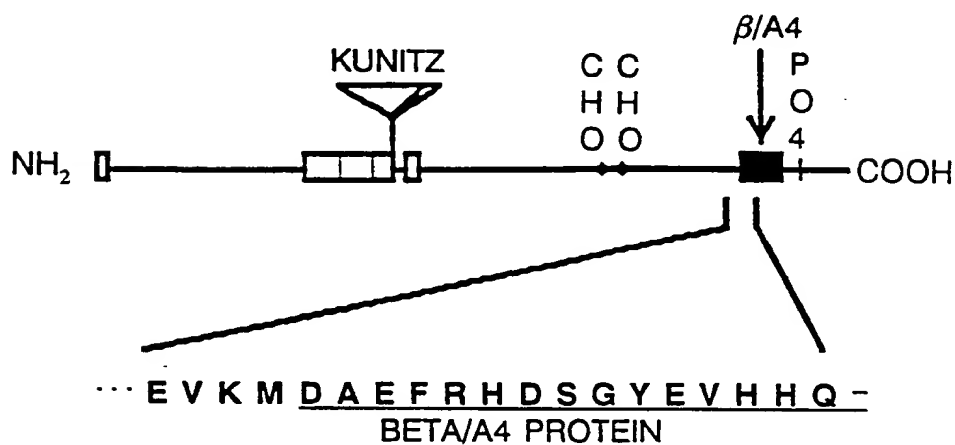
PROTEASE SUBSTRATES:

CHROMOGENIC: MeOSuc- EVKM -paranitroanilide

JUNCTION PEPTIDE: EVKMDAEFRHDSGYEVHHQ

FIG. 5

Brain chymase, but not cathepsin G or chymotrypsin, hydrolyzes
the APP substrate mimic MeOSuc-Glu-Val-Lys-Met-pNa (EVKM)
and related substrates
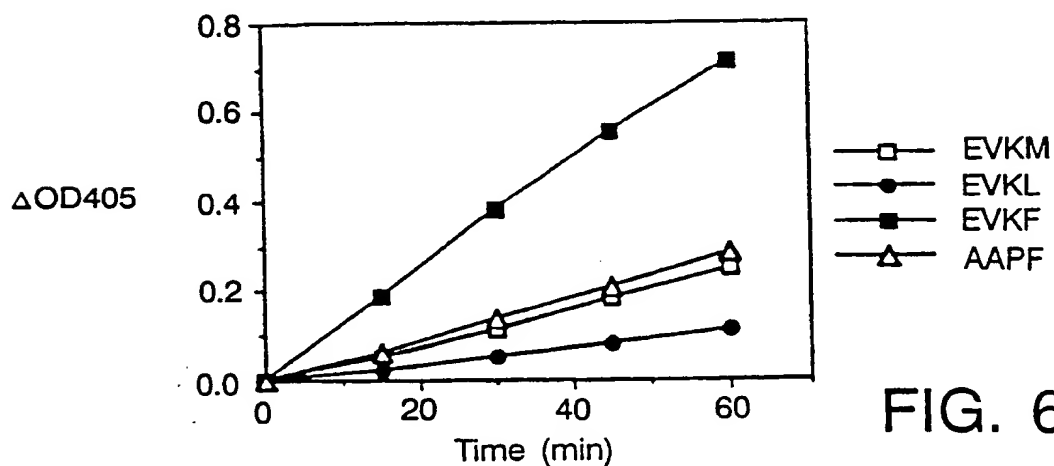
FIG. 6a
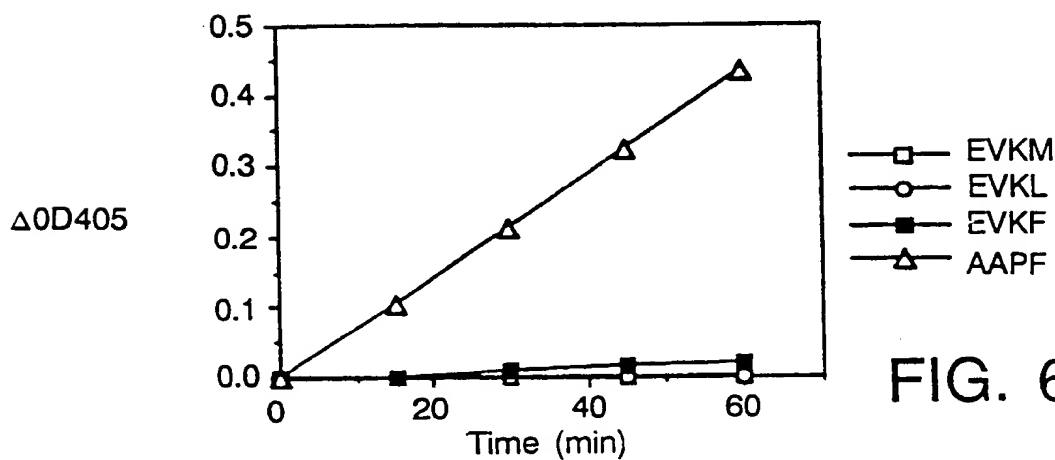
FIG. 6b
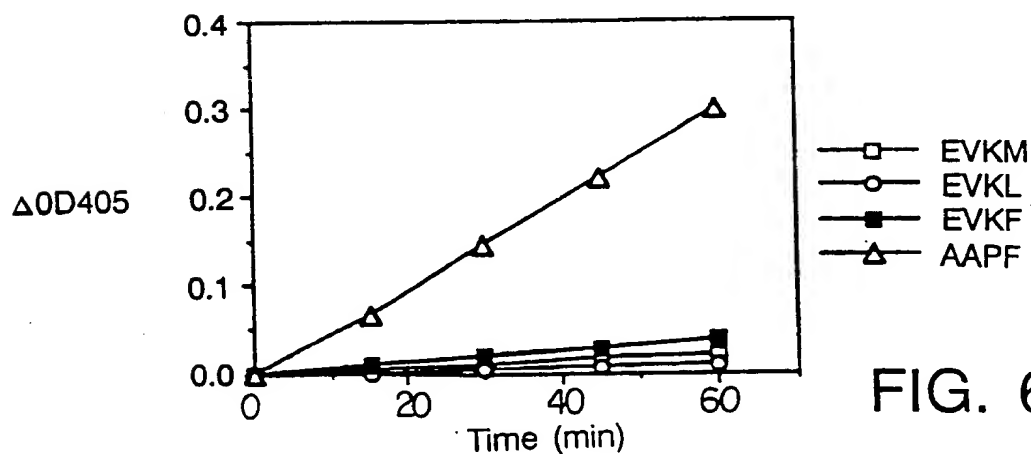
FIG. 6c

SUBSTITUTE SHEET
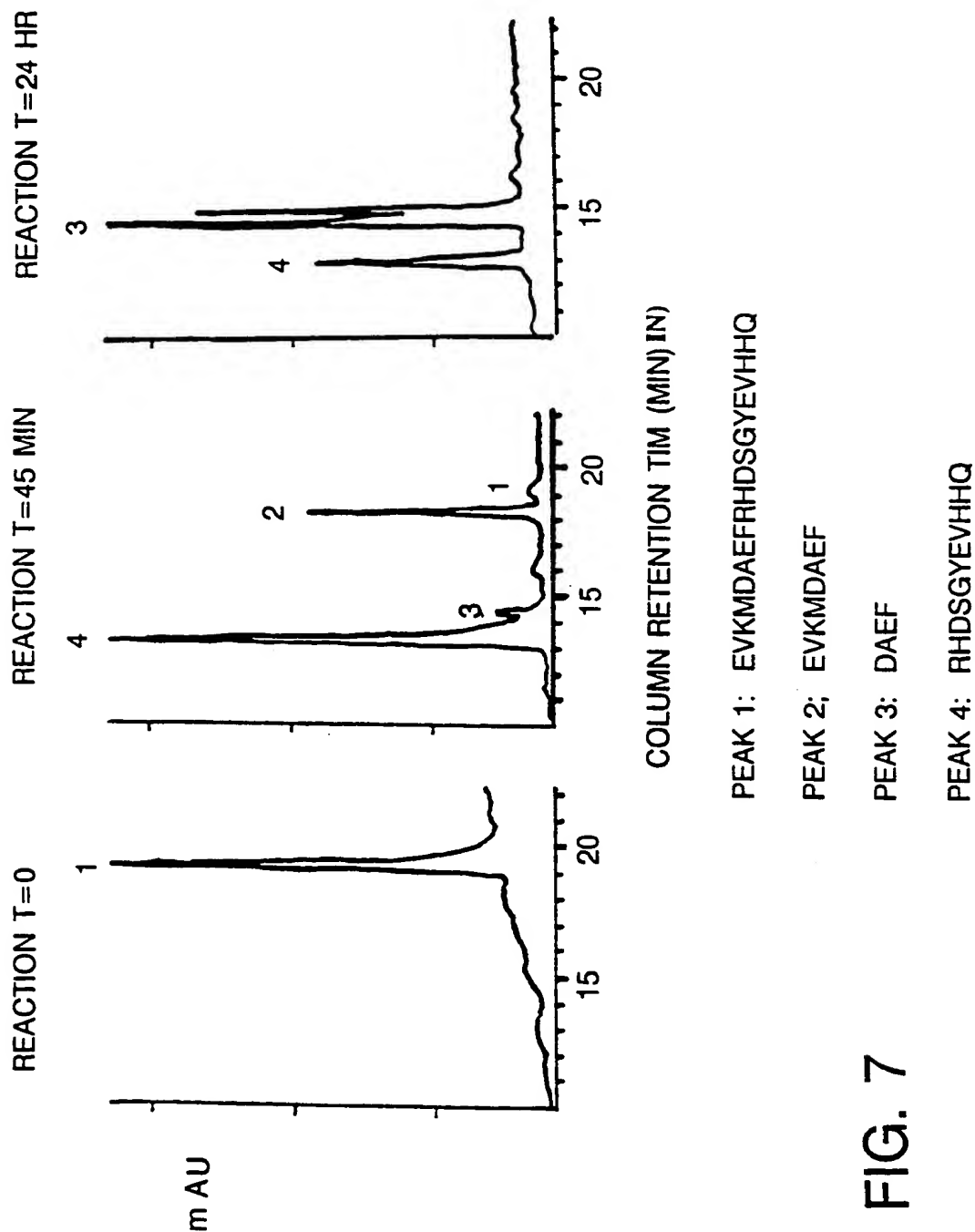
REACTION T=24 HR

REACTION T=45 MIN

REACTION T=0

COLUMN RETENTION TIM (MIN) IN)

PEAK 1: EVKMDAEFRHDSGYEVHHQ

PEAK 2; EVKMDAEF

PEAK 3: DAEF

PEAK 4: RHDSGYEVHHQ

Chymase degrades an APP-mimic junction peptide
between Met and Asp.

m AU

FIG. 7
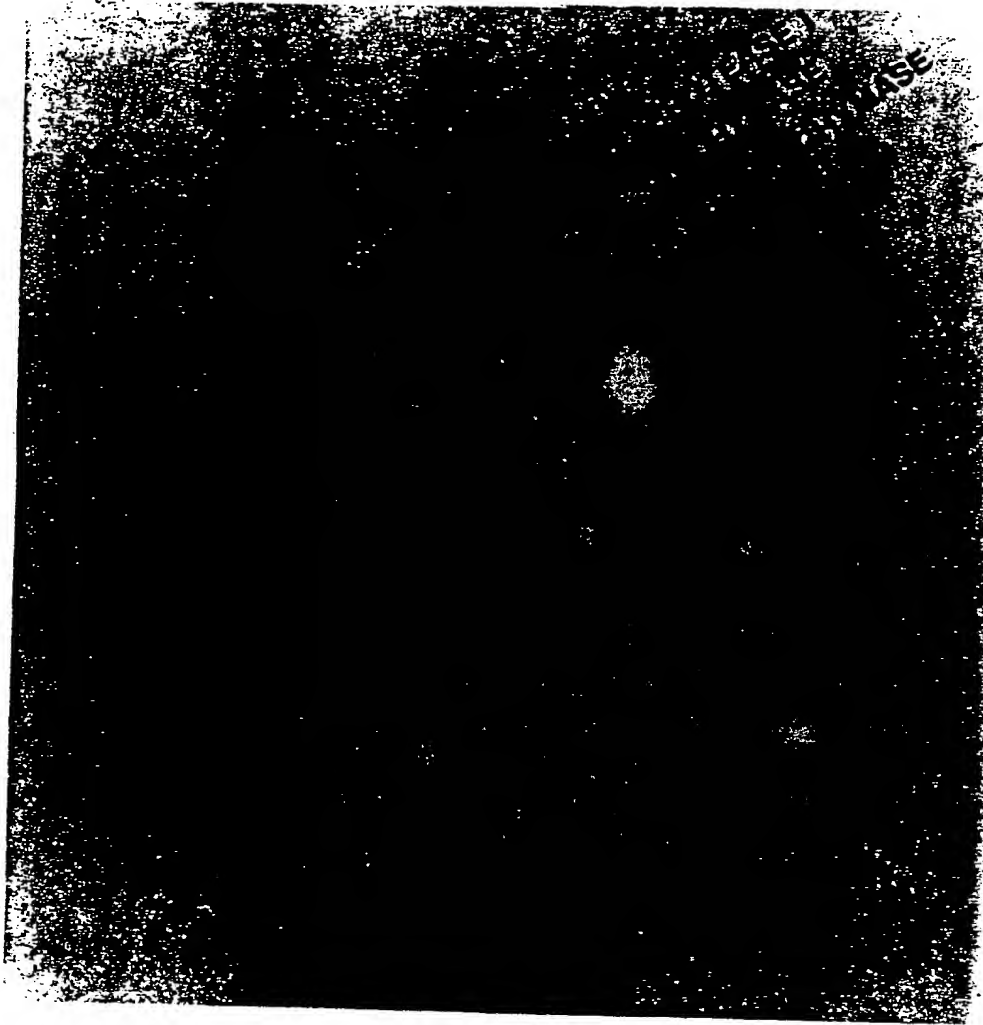
FIG. 8

PURIFICATION STEP	CHYMO-LIKE ACTIVITY (U)	TOTAL PROTEIN (MG)	FOLD PURIFICATION
1. HOMOGENATE		9000	
2. DETERGEN-INSOLUBLE		4600	
3. WATER-INSOLUBLE		3750	
4. HIGH SALT EXTRACTION	5200	525	1
5. HEPARIN-SEPHAROSE	3100	6.0	52
6. TRYPSIN INHIBITOR-AGAROSE	765	0.070	1100

FIG. 9

PURIFICATION ON LIMA BEAN
TRYPSIN INHIBITOR-AGAROSE
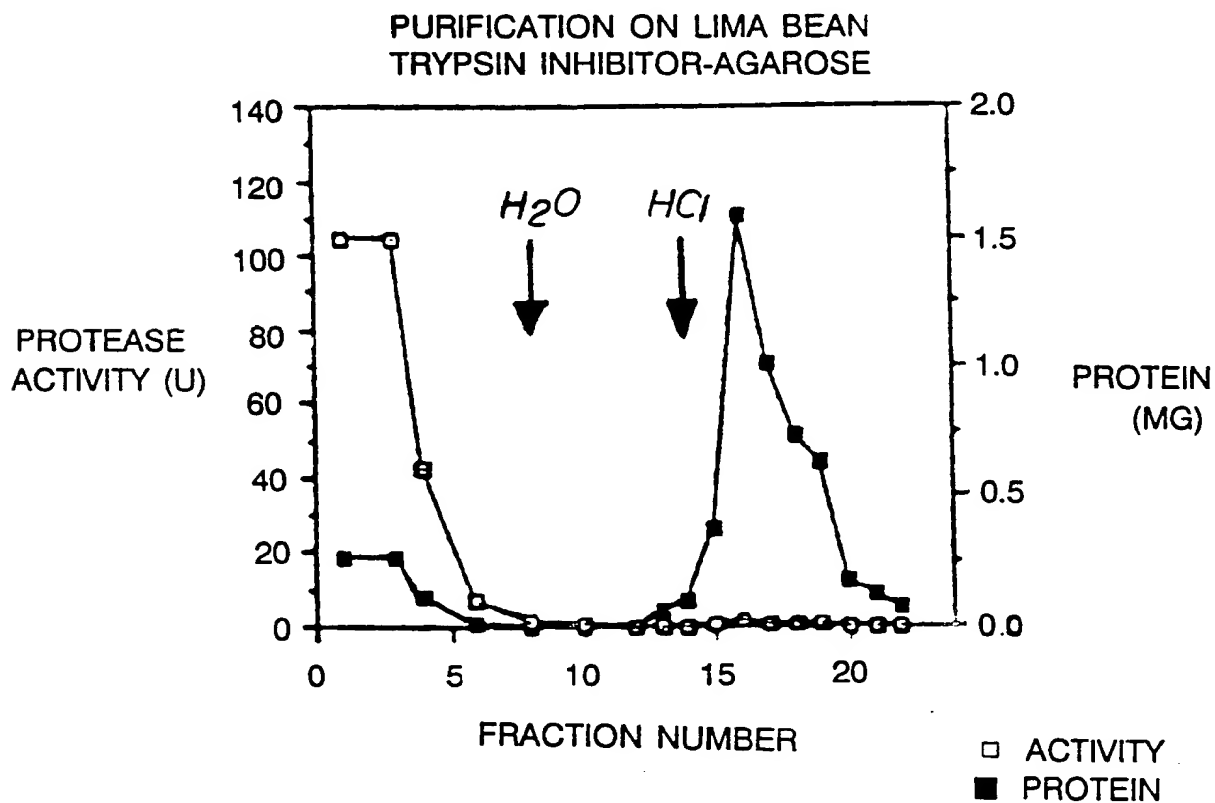
FIG. 10

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FIG. 11   Rat brain chymase para-nitroanilide substrates

	P5	P4	P3	P2	P1	relative kcat/Km
1.	MeOSuc	Glu	Val	Lys	Phe	1000
2.	MeOSuc	Glu	Val	Lys	Met	40
3.	MeOSuc	Glu	Val	Lys	Leu	41
4.	MeOSuc	Glu	Val	Lys	d-Phe	<1
5.	MeOSuc	Glu	Val	Lys	Nle	124
6.	Suc	Ala	Ala	Pro	Phe	69
7.	Suc	Ala	Ala	Pro	Met	6
8.	Suc	Ala	Ala	Pro	Val	<1
9.	Suc	Ala	Ala	Pro	Ala	<1
10.		MeOSuc	Glu	Val	Lys	<1
11.		Sub	Val	Lys	Phe	1304
12.		MeOSub	Val	Lys	Phe	959
13		Az	Val	Lys	Phe	1235
14.		MAz	Val	Lys	Phe	377
15.		MAd	Val	Lys	Phe	1166
16.		Ad	Val	Lys	Phe	2213
17.		d-Glu	Val	Lys	Phe	546
18.		Isobutyryl	Glu	Leu	Phe	230
19.		Ac	Glu	Leu	Phe	240
20.	l-Ser	Glu	Val	Lys	Phe	747
21.	d-Ser	Glu	Val	Lys	Phe	848
22.	Boc-d-Ser	Glu	Val	Lys	Phe	855
23.	MeOSuc-Ser	Glu	Val	Lys	Phe	908
24.	MeOSuc-Ile-Ser	Glu	Val	Lys	Phe	1361
25.	Ac	Glu	Val	Lys	Phe	573
26.	Biotin	Glu	Val	Lys	Phe	158
27.	Z	Glu	Val	Lys	Phe	887
28.	Ac	Ala	Ala	Pro	Phe	41
29.		Suc	Ala	Ala	Phe	56
30.			Ala	Ala	Phe	32
31.		Suc	Phe	Leu	Phe	434
32.		Suc	Val	Lys	Phe	1060
33.		Suc	Gly	Gly	Phe	8
34.		MeOSuc	Val	Lys	Phe	414
35.		MeOSuc	Glu	Lys	Phe	255
36.	MeOSuc	Glu	Phe	Lys	Phe	361
37.	MeOSuc	Glu	Phe	Glu	Phe	21
38.	MeOSuc	Glu	Val	Orn	Phe	586
39.	MeOSuc	Glu	Val	Arg	Phe	622
40.	MeOSuc	Glu	Val	Nle	Phe	300
41.	MeOSuc	Glu	Val	Phe	Phe	214
42.	MeOSuc	Glu	Val	Pro	Phe	493
43.	MeOSuc	Glu	Val	Leu	Phe	1144
44.	MeOSuc	Asp	Val	Lys	Phe	947
45.	MeOSuc	Val	Glu	Lys	Phe	447
46.	MeOSuc	Lys	Glu	Lys	Phe	328
47.	MeOSuc	Glu	Ala	Lys	Phe	551
48.	MeOSuc	Glu	Gly	Lys	Phe	14
49.	MeOSuc	Glu	Aib	Lys	Phe	<10
50.	MeOSuc	Glu	Nle	Lys	Phe	658
51.	Suc	Ala	Lys	Pro	Phe	26
52.	Suc	Ala	Gln	Pro	Phe	201
53.	Suc	Ala	Glu	Pro	Phe	217
54.	Suc	Ala	Phe	Pro	Phe	190

SUBSTITUTE SHEET
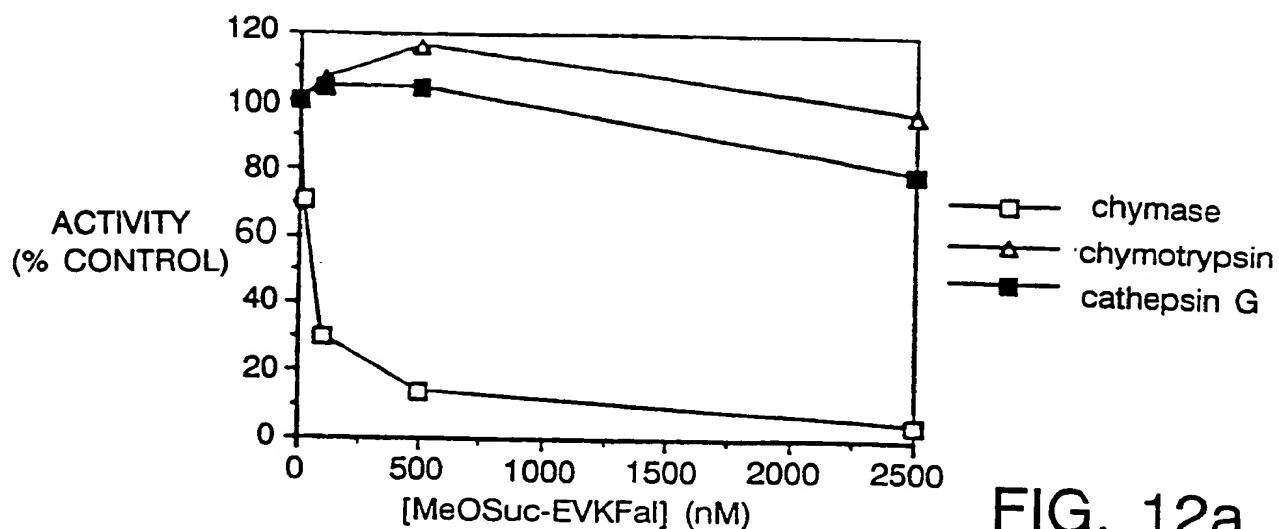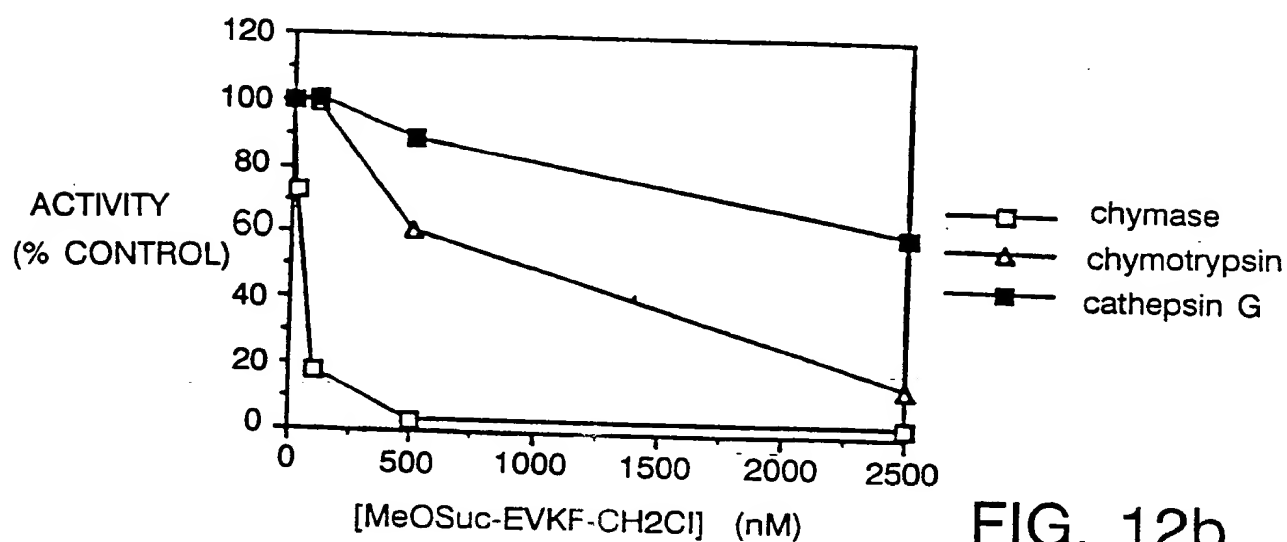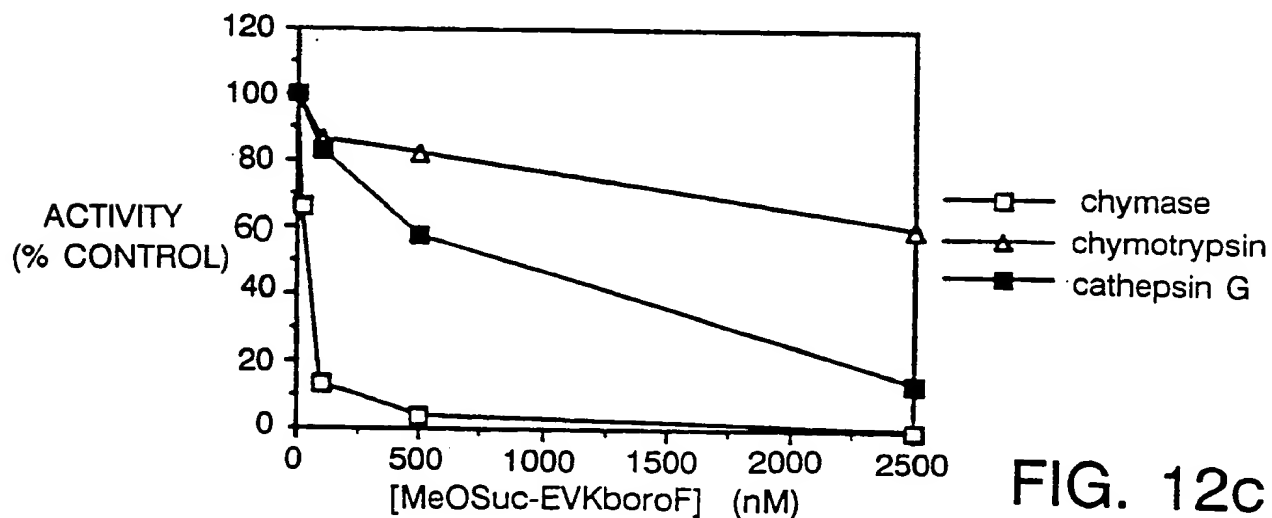
FIG. 12a

FIG. 12b

FIG. 12c

Inhibition of various proteases
by MeOSuc-Glu-Val-Lys-Phe-aldehyde

protease	%inhibition at 10 uM
chymase, rat brain	Ki=15 nM
multicatalytic protease, human brain	92
elastase, porcine pancreas	0
thrombin, human plasma	0
plasmin, human plasma	80
cathepsin B, bovine spleen	83
urokinase, human kidney	15
trypsin, bovine pancrease	20
kallikrein, human plasma	0

FIG. 13a

INHIBITION OF MULTICATALYTIC PROTEASE
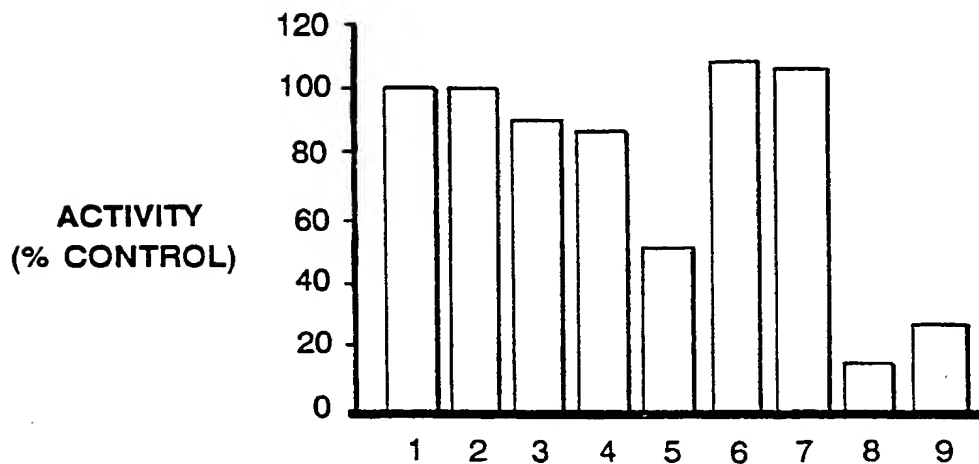
ACTIVITY
(% CONTROL)

1 NO INHIBITOR
2 ANTICHYMOTRYPSIN, 5 ug/ml
3 PHENYLMETHYSULFONYL FLUORIDE, 100 uM
4 LEUPEPTIN, 50 uM
5 N-ETHYLAMALEIMIDE, 1 mM
6 PEPSTATIN, 10 uM
7 EDTA, 1 mM
8 MeOSuc-EYKFal, 10 uM
9 MeOSuc-EYKboroF, 10 uM

FIG. 13b

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FIG. 14   Multicatalytic Protease Activity: % relative hydrolysis

	P 5	P 4	P 3	P 2	P 1	Hydrolysis
1.	MeOSuc	Glu	Val	Lys	Met	100
2.	MeOSuc	Glu	Val	Lys	Phe	104
3.	MeOSuc	Glu	Val	Lys	d-Phe	0
4.	MeOSuc	Glu	Val	Lys	Nle	110
5.	MeOSuc	Glu	Val	Lys	Leu	158
6.		MeOSuc	Glu	Val	Lys	0
7.	MeOSuc	Glu	Val	Pro	Phe	0
8.	MeOSuc	Glu	Val	Arg	Phe	432
9.	MeOSuc	Glu	Val	Orn	Phe	163
10.	MeOSuc	Glu	Val	Glu	Phe	0
11.	MeOSuc	Glu	Val	Phe	Phe	137
12.	MeOSuc	Glu	Val	Leu	Phe	70
13.	MeOSuc	Glu	Val	Nle	Phe	82
14.	Suc	Ala	Ala	Pro	Phe	0
15.	Suc	Ala	Ala	Pro	Met	0
16.		Suc	Gly	Gly	Phe	2
17.	MeOSuc	Glu	Phe	Lys	Phe	162
18.	MeOSuc	Glu	Ala	Lys	Phe	51
19.	MeOSuc	Glu	Nle	Lys	Phe	66
20.	MeOSuc	Glu	Gly	Lys	Phe	5
21.	MeOSuc	Lys	Val	Lys	Phe	32
22.	MeOSuc	Val	Glu	Lys	Phe	125
23.		MeOSuc	Ile	Lys	Phe	272
24.		MeOSuc	Gly	Lys	Phe	29
25.		Isobutyryl	d-Glu	Lys	Phe	0
26.			d-Glu	Lys	Phe	0
27.	Suc	Glu	Aib	Lys	Phe	0
28.		MeOSuc	Ala	Lys	Phe	27
29.		MeOSuc	Pro	Lys	Phe	10
30.		MeOSuc	d-Pro	Lys	Phe	0
31.			Ad	Lys	Phe	20
32.			Az	Lys	Phe	38
33.		MeOSuc	Val	Lys	Phe	155
34.		Suc	Val	Lys	Phe	80
35.		MeOAz	Val	Lys	Phe	81
36.		Az	Val	Lys	Phe	54
37.		MeOSub	Val	Lys	Phe	127
38.		Sub	Val	Lys	Phe	107
39.		MeOAd	Val	Lys	Phe	207
40.		Ad	Val	Lys	Phe	228
41.		Z	Val	Lys	Phe	82
42.		Boc	Ala	Ala	Phe	69
43.		Suc	Ala	Ala	Phe	11
44.			Ala	Ala	Phe	14
45.	Z	Glu	Val	Lys	Phe	478
46.	d-Ser	Glu	Val	Lys	Phe	64
47.	Biotin	Glu	Val	Lys	Phe	10
48.	Ac	Glu	Val	Lys	Phe	66

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FIG. 15

MeOSuc-Glu-Val-Lys-NH-CH-CHO
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad$ CH2C6H5

MeOSuc-Ser-Glu-Val-Lys-NH-CH-B $\overset{\diagup \text{OH}}{\underset{\diagdown \text{OH}}{}}$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ CH2C6H5

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ O
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ ||
MeOSuc-Glu-Val-Lys-NH-CH-C-CH2C1
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad$ CH2C6H5

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ O
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ ||
MeOSuc-Glu-Val-Lys-NH-CH-C-CH3
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad$ CH2C6H5

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ O
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ ||
MeOSuc-Glu-Val-Lys-NH-CH-C-CF3
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad$ CH2C6H5

MeOSuc-Glu-Val-Lys-NH-CH-CHO
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad$ (CH2)3-CH3

MeOSuc-Ser-Glu-Val-Lys-NH-CH-B $\overset{\diagup \text{OH}}{\underset{\diagdown \text{CH}}{}}$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ (CH2)3-CH3

MeOSuc-Ser-Glu-Val-Lys-NH-CH-B $\overset{\diagup \text{0-CH-(CH3)2}}{\underset{\diagdown \text{0-CH-(CH3)2}}{}}$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ CH2
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ C6H5

D-Ser-Glu-Val-Lys-NH-CH-B $\overset{\diagup \text{O-CH-(CH3)2}}{\underset{\diagdown \text{O-CH-(CH3)2}}{}}$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad$ CH2
$\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad$ C6H5

MeOSuc-Ser-Glu-Val-Lys-NH-CH-B $\overset{\diagup \text{O-CH2CH2}}{\underset{\diagdown \text{O-CH2CH2}}{}}$NH
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ CH2
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad\quad\quad\quad$ C6H5

D-Glu-Val-Lys-NH-CH-B $\overset{\diagup \text{O-CH-(CH3)2}}{\underset{\diagdown \text{O-CH-(CH3)2}}{}}$
$\quad\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad$ CH2
$\quad\quad\quad\quad\quad\quad$ |
$\quad\quad\quad\quad\quad\quad$ C6H5

FIG. 16a

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MeOSuc-Ser-Glu-Val-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

Suc-Ile-Ser-Glu-Val-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

Suc-Lys-Thr-Glu-Glu-Ile-Ser-Glu-Val-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　　　　　　　　　　|
　　　　　　　　　　　　　　　　　　CH2C6H5

D-Lys-Thr-Gly-Glu-Ile-Ser-Glu-Val-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　　　　　　　　　|
　　　　　　　　　　　　　　　　　CH2C6H5

MeOSuc-Ser-Glu-Val-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2CH(CH3)2

MeOSuc-Ser-Glu-Val-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2-(CH2)2CH3

MeOSuc-Ser-Glu-Val-Arg-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

MeOSuc-Ser-Glu-Val-Cit-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

MeOSuc-Ser-Glu-Val-Harg-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

MeOSuc-Ser-Glu-Leu-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

MeOSuc-Ser-Glu-Ile-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

MeOSuc-Ser-Glu-Nva-Lys-NH-CH-B$\overset{\displaystyle OH}{\underset{\displaystyle OH}{\diagup}}$
　　　　　　　　　　　|
　　　　　　　　　　CH2C6H5

FIG. 16b

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MeOSuc-Ser-Glu-Nle-Lys-NH-CH-B$\overset{\nearrow OH}{\searrow}$ OH
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;\;CH2C6H5$

MeOSuc-Ser-Asp-Val-Lys-NH-CH-B$\overset{\nearrow OH}{\searrow}$ OH
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;\;|$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;\;\;CH2C6H5$

MeOSuc-Thr-Glu-Val-Lys-NH-CH-B$\overset{\nearrow OH}{\searrow}$ OH
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;\;CH2C6H5$

nC18H37C-D-Ser-Glu-Val-Lys-NH-CH-B$\overset{\nearrow O\text{-}CH\text{-}(CH3)2}{\searrow O\text{-}CH\text{-}(CH3)2}$
$\quad\quad\;\;||\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad|$
$\quad\quad\;\;O\quad\quad\quad\quad\quad\quad\quad\quad\quad\;CH2$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;C6H5$

nC18H37C-D-Ser-Glu-Val-Lys-NH-CH-CH0
$\quad\quad\;\;||\quad\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\;\;O\quad\quad\quad\quad\quad\quad\quad\quad\;\;CH2$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;C6H5$

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;O$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;||$
nC18H37C-D-Ser-Glu-Val-Lys-NH-CH-C-CH2C1
$\quad\quad\;\;||\quad\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\;\;O\quad\quad\quad\quad\quad\quad\quad\quad\;\;CH2$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;C6H5$

DNS-Ser-Glu-Val-Lys-NH-CH-B$\overset{\nearrow O\text{-}CH\text{-}(CH3)2}{\searrow O\text{-}CH\text{-}(CH3)2}$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\quad\;CH2$
$\quad\quad\quad\quad\quad\quad\quad\quad\;C6H5$

$\quad\quad\quad\quad O$
$\quad\quad\quad\quad||$
C6H5CH2OC-Ser-Glu-Val-Lys-NH-CH-B$\overset{\nearrow O\text{-}CH\text{-}(CH3)2}{\searrow O\text{-}CH\text{-}(CH2)2}$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;CH2$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;C6H5$

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\;O\;O$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\;||\;||$
D-Ser-Glu-Val-Lys-NH-CH-C-C-CH3
$\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\;CH2C6H$

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\;O\;O$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\;||\;||$
D-Ser-Glu-Val-Lys-NH-CH-C-C-OCH3
$\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\;CH2C6H5$

$\quad\quad\quad\quad\quad\quad\quad\quad\quad\;O$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\;||$
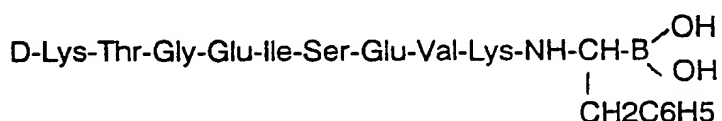D-Ser-Glu-Val-Lys-NH-CH-C-CF3
$\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\;CH2C6H5$

FIG. 16c

D-Pglu-Ile-Ser-Glu-Val-Lys-NH-CH-B$\overset{\nearrow 0\text{-}C(CH3)2}{\searrow 0\text{-}C(CH3)2}$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;|$
$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad\;CH2C6H5$

D-Glu-Val-Lys-NH-CH-CHO
$|$
CH2C6H5

Sub-Val-Lys-NH-CH-B$\overset{\nearrow OH}{\searrow OH}$
$|$
CH2C6H5

$\overset{O}{\overset{\|}{}}$
Az-Val-Lys-NH-CH-C-CH2C1
$|$
CH2C6H5

$\overset{O}{\overset{\|}{}}$
Ad-Val-Orn-NH-CH-C-CH3
$|$
CH2C6H5

$\overset{O}{\overset{\|}{}}$
MeOSuc-Glu-Val-Orn-NH-CH-C-CF3
$|$
CH2C6H5

Suc-Val-Orn-NH-CH-CHO
$|$
(CH2)3-CH3

Ad-Val-Arg-NH-CH-B$\overset{\nearrow OH}{\searrow OH}$
$|$
(CH2)3-CH3

Ad-Val-Glu-Arg-NH-CH-B$\overset{\nearrow O\text{-}CH\text{-}(CH3)2}{\searrow O\text{-}CH\text{-}(CH3)2}$
$|$
CH2
$|$
C6H5

Ad-Val-Gln-Arg-NH-CH-B$\overset{\nearrow O\text{-}CH\text{-}(CH3)2}{\searrow O\text{-}CH\text{-}(CH3)2}$
$|$
CH2
$|$
C6H5

Ad-Val-Phe-HArg-NH-CH-B$\overset{\nearrow O\text{-}CH2CH2}{\searrow O\text{-}CH2CH2}$NH
$|$
CH2
$|$
C6H5

Glu-Ala-Nle-NH-CH-B$\overset{\nearrow O\text{-}CH\text{-}(CH3)2}{\searrow O\text{-}CH\text{-}(CH3)2}$
$|$
CH2
$|$
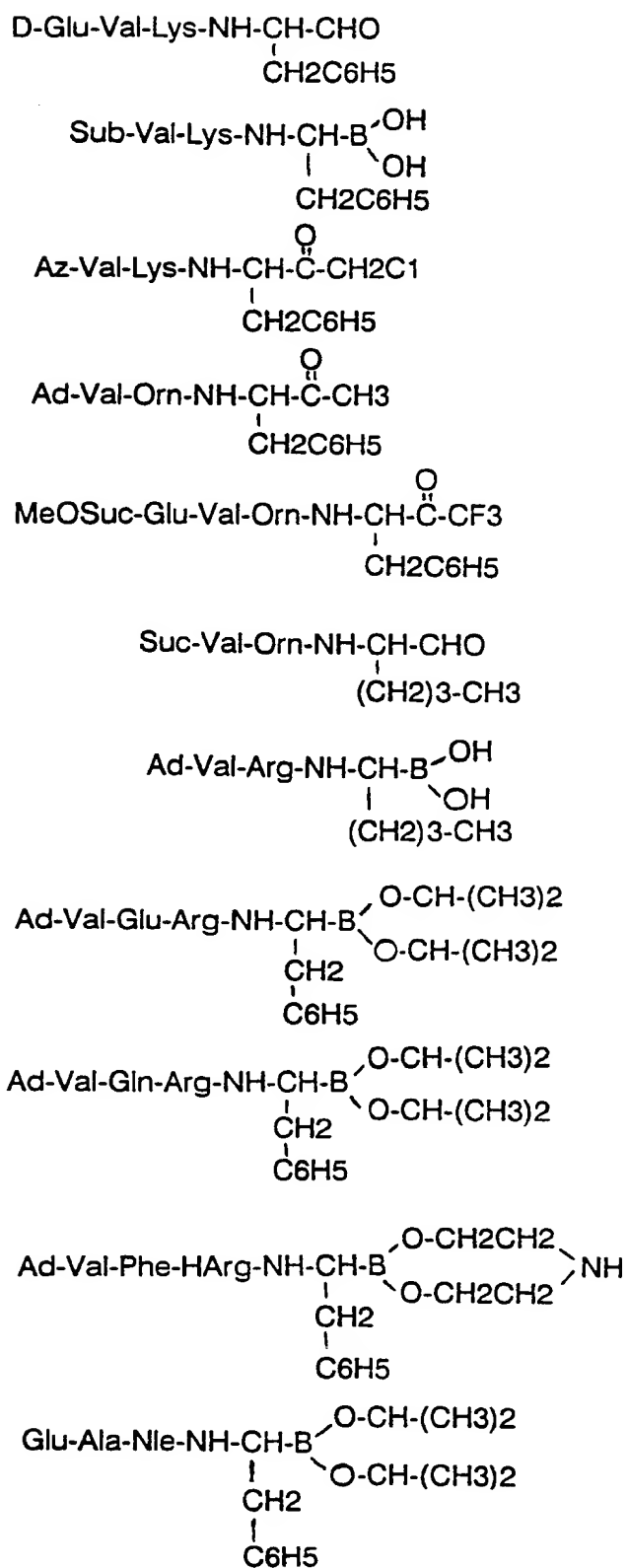C6H5

FIG. 16d

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```
                                    OH
                                   ╱
Ad-Glu-Val-Arg-NH-CH-B
                      |            ╲
                      |              OH
                    CH2C6H5


                                   OH
                                  ╱
D-Glu-Val-Orn-NH-CH-B
                     |            ╲
                     |              OH
                   CH2C6H5


                                        OH
                                       ╱
D-Ile-Ser-Glu-Val-Lys-NH-CH-B
                            |          ╲
                            |            OH
                          CH2C6H5

                                         OH
                                        ╱
Ac-Ile-Ser-Asp-Val-Arg-NH-CH-B
                             |          ╲
                             |            OH
                           CH2C6H5


                               OH
                              ╱
Sub-Val-Arg-NH-CH-B
                  |           ╲
                  |             OH
                CH2CH(CH3)2


                            OH
                           ╱
Az-Val-Orn-NH-CH-B
                 |         ╲
                 |           OH
               CH2-(CH2)2CH3


                              OH
                             ╱
Ad-Val-HArg-NH-CH-B
                  |          ╲
                  |            OH
                CH2C6H5


                            OH
                           ╱
Sub-Val-Lys-NH-CH-B
                 |         ╲
                 |           OH
               CH2C6H5


                           OH
                          ╱
Ad-Val-Arg-NH-CH-B
                 |        ╲
                 |          OH
               CH2C6H5


                           OH
                          ╱
Ad-Val-Arg-NH-CH-B
                 |        ╲
                 |          OH
               CH2C6H5


                                    OH
                                   ╱
MeOSuc-Glu-Leu-Lys-NH-CH-B
                         |         ╲
                         |           OH
                       ·CH2C6H5


                               OH
                              ╱
Z-Glu-Nle-Orn-NH-CH-B
                    |         ╲
                    |           OH
                  CH2C6H5
```
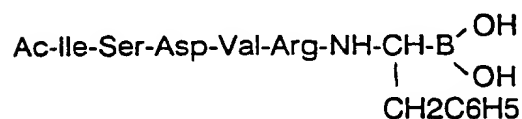
FIG. 16e

SUBSTITUTE SHEET

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Az-Val-Lys-NH-CH-B$\diagup^{OH}_{\diagdown OH}$
　　　　　　　|
　　　　　CH2C6H5

Ad-Val-Arg-NH-CH-B$\diagup^{OH}_{\diagdown OH}$
　　　　　　　|
　　　　　CH2C6H5

Z-Glu-Val-Orn-NH-CH-B$\diagup^{OH}_{\diagdown OH}$
　　　　　　　　　|
　　　　　　　CH2C6H5

D-Glu-Leu-HArg-NH-CH-B$\diagup^{O-CH-(CH3)2}_{\diagdown O-CH-(CH3)2}$
　　　　　　　　　　|
　　　　　　　　　CH2
　　　　　　　　　C6H5

Az-Val-Lys-NH-CH-CHO
　　　　　　　　|
　　　　　　　CH2
　　　　　　　C6H5

　　　　　　　　　　　O
　　　　　　　　　　　‖
Ad-Val-Arg-NH-CH-C-CH2C1
　　　　　　　　|
　　　　　　　CH2
　　　　　　　C6H5

Ad-Ser-Glu-Val-Orn-NH-CH-B$\diagup^{OH-CH-(CH3)2}_{\diagdown OH-CH-(CH3)2}$
　　　　　　　　　　　　|
　　　　　　　　　　　CH2
　　　　　　　　　　　C6H5

Z-Glu-Leu-Lys-NH-CH-B$\diagup^{OH-CH-(CH3)2}_{\diagdown OH-CH-(CH3)2}$
　　　　　　　　　|
　　　　　　　　CH2
　　　　　　　　C6H5

　　　　　　　　　　　O O
　　　　　　　　　　　‖ ‖
Ac-Glu-Val-Lys-NH-CH-C-C-CH3
　　　　　　　　　|
　　　　　　　　CH2C6H5

　　　　　　　　　　O O
　　　　　　　　　　‖ ‖
D-Glu-Nle-Arg-NH-CH-C-C-OCH3
　　　　　　　　　|
　　　　　　　　CH2C6H5

　　　　　　　　　O
　　　　　　　　　‖
Ad-Ile-Arg-NH-CH-C-CF3
　　　　　　　　|
　　　　　　　CH2C6H5

Az-Ile-Orn-NH-CH-B$\diagup^{O-C(CH3)2}_{\diagdown O-C(CH3)2}$
　　　　　　　　|
　　　　　　　CH2C6H5

FIG. 16f

SUBSTITUTE SHEET

$$HO-\overset{O}{\overset{\|}{C}}-CH-\overset{O}{\overset{\|}{C}}-Lys-NH-CH-CHO$$
$$\quad\quad\quad CH(CH3)2 \quad\quad CH2C6H5$$

$$CH3-(CH2)3-CH-CH2-C-Arg-NH-CH-B\overset{OH}{\underset{OH}{<}}$$
$$\quad\quad\quad\quad CH2-C6H5 \quad\quad\quad CH2C6H5$$

$$HOO-CH2-CH-CH2-\overset{O}{\overset{\|}{C}}-Orn-NH-CH-\overset{O}{\overset{\|}{C}}-CH2C1$$
$$\quad\quad\quad CH2(CH3)2 \quad\quad\quad CH2C6H5$$

$$CH3-(CH2)4-CH-\overset{O}{\overset{\|}{C}}-HArg-NH-CH-C-CH3$$
$$\quad\quad\quad\quad CH2(CH3)2 \quad\quad CH2C6H5$$

$$CH3O-\overset{O}{\overset{\|}{C}}-CH-\overset{O}{\overset{\|}{C}}-Arg-NH-CH-\overset{O}{\overset{\|}{C}}-CF3$$
$$\quad\quad\quad CH2C6H5 \quad\quad\quad CH2C6H5$$

$$HOOC-CH2-CH-CH2-\overset{O}{\overset{\|}{C}}-Lys-NH-CH-CHO$$
$$\quad\quad\quad CH2-CH2(CH3)2 \quad (CH2)3-CH3$$

$$HOOC-CH2-CH-C-Arg-NH-CH-B\overset{OH}{\underset{OH}{<}}$$
$$\quad\quad\quad CH(CH3)2 \quad\quad\quad (CH2)3-CH3$$

$$HOOC-CH-(CH2)2-C-Orn-NH-CH-B\overset{O-CH-(CH2)2}{\underset{O-CH-(CH2)2}{<}}$$
$$\quad\quad CH2(CH3)2 \quad\quad\quad CH2$$
$$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad C6H5$$

$$HOOC-CH2-CH-CH2-C-Arg-NH-CH-B\overset{O-CH-(CH3)2}{\underset{O-CH-(CH3)2}{<}}$$
$$\quad\quad CH2-CH(CH3)3 \quad\quad\quad CH2$$
$$\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad C6H5$$

$$CH3O-C-CH-C-Lys-NH-CH-B\overset{O-CH2CH2}{\underset{O-CH2CH2}{<}}NH$$
$$\quad\quad CH(CH3)2 \quad CH2$$
$$\quad\quad\quad\quad\quad\quad\quad C6H5$$

$$HO-C-CH-C-Lys-NH-CH-B\overset{O-CH-(CH3)2}{\underset{O-CH-(CH3)2}{<}}$$
$$\quad\quad\quad\quad\quad\quad\quad\quad CH2$$
$$\quad\quad\quad\quad\quad\quad\quad\quad C6H5$$

$$CH2CH2CH(CH3)2$$

FIG. 16g

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MeoSuc-Glu-Val-Lys-AzaPhe-OCH3

Ad-Val-Arg-AzaPhe-OC6H5-NO2

Sub-Val-Orn-AzaPhe-NH2

Z-Glu-Val-Lys-AzaPhe-Glu-NH2

Ad-Phe-Arg-AzaPhe-Asp-OH

Sub-Phe-Orn-AzaPhe-Asp-Ala-NH2

MeOSuc-Val-Lys-AzaPhe-Asp-Ala-Glu-Phe-OH

Z-Glu-Val-Phe-AzaLeu-OCH3

Ad-Val-Lys-AzaLeu-Asp-Ala-Glu-NH2

Sub-Phe-Lys-AzaMet-Asp-Ala-NH2

Ac-Glu-Val-Arg-AzaNle-Asp-Ala-NH2

Ad-Val-Orn-AzaNle-Asp-NH2

FIG. 16h

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/01474

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07K 3/00; A23J 1/00; C12Q 1/37
U.S. CL.: 530/350; 435/24; 530/412

II. FIELDS SEARCHED

	Minimum Documentation Searched 7
Classification System	Classification Symbols
US.	530/350, 412; 435/24

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

Databases: USPTO Automated Patent System STN

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
Y	Fluka Catalog 1988/89 (Canada) Fluka Chemical Corporation. pages 367 and 368: see entire document.	1-5 39-42
Y,P	US.A. 4.997.929 (Collins et al) 05 March 1991. See the entire document.	1-5. 39-42

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 April 1991	20 JUN 1991
International Searching Authority	Signature of Authorized Officer
ISA/US	Bradley L. Sisson

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SEC ND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (b) for the following reasons:

1. ☐ Claim numbers   . because they relate to subject matter [12] not required to be searched by the Authority, namely:

2. ☐ Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attaachment.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-5, 39-42

           Telephone practice

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

(12) EUROPEAN PATENT APPLICATION

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

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Bayer AG Konzernverwaltung RP Patente
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D-51368 Leverkusen (DE)

(54) Methods for detecting beta amyloid precursor protein processing enzymes.

(57) Disclosed are methods for regulating formation of beta-amyloid protein with inhibitors of proteases specific for the Precursor to the Alzheimer's Disease beta-amyloid protein, such as inhibitors of aspartic protease, cathepsin D, and a chymotryptic-like serine protease ; a method for detecting in vitro protease activity for molecules which possess specificity necessary for the degradation of the Amyloid Precursor Protein ; and assays for identifying inhibitors of proteases specific for the Amyloid Precursor Protein.

EP 0 569 777 A2

FIELD OF THE INVENTION

The invention relates to methods for identifying proteolytic enzymes with specificity for processing the precursor to the Alzheimer's Disease beta-amyloid protein; methods for identifying inhibitors of proteases specific
5   for the precursor to the beta-amyloid protein; and methods for regulating formation of beta-amyloid protein with inhibitors of proteases specific for the precursor to the beta-amyloid protein, such as inhibitors of aspartic protease, cathepsin D, and a chymotryptic-like serine protease.

BACKGROUND

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The present assays have utility in the identification of the proteases which control the rate of formation of amyloidic peptides in the brains of Alzheimer's Disease patients. As such, they can be used to isolate such proteases, and can also be used to identify protease inhibitors which can be used as therapeutics for Alzheimer's Disease. Described hereinbelow is the application of the assays to identify the aspartic protease, cath-
15   epsin D as a major amyloidogenic, protease for processing Amyloid Precursor Protein (hereinafter "APP"). Also provided is a partial characterization of a second, serine protease which can form amyloidic precursors from the APP holoprotein.

Alzheimer's Disease (hereinafter also abbreviated to "AD") is a progressive, degenerative disorder of the brain, characterized by progressive atrophy, usually in the frontal, parietal and occipital cortices. The clinical
20   manifestations of AD include progressive memory impairments, loss of language and visuospatial skills, and behavioral deficits (McKhan et al., 1986, Neurology $\underline{34}$:939). Overall cognitive impairment is attributed to degeneration of neuronal cells located throughout the cerebral hemispheres (Price, 1986, Annu. Rev. Neurosci. $\underline{9}$:489).

Pathologically, the primary distinguishing features of the post-mortem brain of an AD patient are, 1) path-
25   ological lesions comprised of neuronal perikarya containing accumulations of neurofibrillary tangles; 2) cerebrovascular amyloid deposits; and 3) neuritic plaques. Both the cerebrovascular amyloid (Wong et al., 1985, PNAS $\underline{82}$:8729) and the neuritic plaques (Masters et al., 1985, PNAS $\underline{82}$:4249) contain a distinctive peptide simply designated, "A4" or "beta-amyloid".

Beta-amyloid is an insoluble, highly aggregating, small polypeptide of relative molecular mass 4,500, and
30   is composed of 39 to 42 amino acids. Several lines of evidence support a role of beta-amyloid in the pathogenesis of AD lesions. For instance, beta-amyloid and related fragments have been shown to be toxic for PC-12 cell lines (Yankner et al., 1989, Science $\underline{245}$:417); toxic for primary cultures of neurons (Yankner et al., 1990, Science $\underline{250}$:279); and cause neuronal degeneration in rodent brains and corresponding amnestic response in the rodents (Flood et al., 1991, PNAS $\underline{88}$:3363; Kowall et al., 1991, PNAS $\underline{88}$:7247).

35   Kang et al., 1987, Nature $\underline{325}$:733, described the beta-amyloid protein as originating from and as a part of a larger precursor protein. To identify this precursor, a full-length complementary DNA clone coding for the protein was isolated and sequenced, using oligonucleotide probes designed from the known beta-amyloid sequence. The predicted precursor contained 695 residues and is currently designated, "APP 695" (Amyloid Precursor Protein 695).

40   Subsequent cloning of the gene encoding the APP proteins revealed that the A4 region was encoded on two adjacent exons (Lemaire et al., 1989, Nucleic Acids Res. $\underline{17}$:517), ruling out the possibility that A4 accumulation is the result of direct expression of an alternatively spliced mRNA. This implied that A4 accumulation must result from abnormal proteolytic degradation of the APP at sites both N- and C-terminal to the peptide region within the APP.

45   It has recently been demonstrated that inheritance patterns in rare forms of Familial Alzheimer's Disease show co-segregation with point mutations within the open reading frame of the APP gene, providing further evidence for a role of beta-amyloid in the pathogenesis of AD. These include mutations C-terminal to the beta-amyloid region (Goate et al., 1991, Nature $\underline{349}$:704; Yoshioka et al., 1991, Biochem Biophys Res Comm, $\underline{178}$:1141; Chartier-Harlin et al., 1991, Nature $\underline{353}$:844; Murrell et al., 1991, Science $\underline{254}$:97), as well as prox-
50   imal to the N-terminus of the beta-amyloid region (Mullan et al., 1992, Nature Genetics $\underline{1}$:345). It has been speculated but not proven that such mutations might increase the rate of beta-amyloid peptide formation in Familial Alzheimer's Disease either by perturbing the normal sorting and or processing of APP; or by altering the recognition sites for specific proteases involved in the generation of beta-amyloid.

APP 695 is the most abundant form of APP found in the human brain, but three other forms exist, APP
55   714, APP 751 and APP 770 (Tanzi et al., 1988, Nature $\underline{351}$:528; Ponte et al., 1988, Nature $\underline{331}$:525; Kitaguchi et al., 1988, Nature $\underline{331}$:530). The different length isoforms arise from alternative splicing from a single APP gene located on human chromosome 21 (Goldgaber et al., 1987, Science $\underline{235}$:877; Tanzi et al., 1987, Science $\underline{235}$:880).

2

APP 751 and APP 770 contain a 56 amino acid Kunitz inhibitor domain, which shares 40% homology with Bovine Pancreatic Trypsin Inhibitor. Both these forms of APP have protease inhibitory activity (Kitaguchi et al., 1988, Nature 311:530; Smith et al., 1990, Science 248:1126), and at least one of these forms is probably what was previously identified as Protease Nexin II (Oltersdorf et al., 1989, Nature 341:144; Van Nostrand et al., 1989, Nature 341:546).

The physiological role for the amyloid precursor proteins has not yet been confirmed. It has been proposed to be a cell surface receptor (Kang et al., 1987, Nature 325:733); an adhesion molecule (Schubert et al., 1989, Neuron 3:689); a growth or trophic factor (Saitoh et al., 1989, Cell 58:615; Araki et al., 1991, Biochem Biophys Res Comm, 181:265; Milward et al., 1992, Neuron 9:129); a regulator of wound healing (Van Nostrand et al., 1990, Science 248:745; Smith et al., 1990, Science 248:1126); or play a role in the cytoskeletal system (Refolo et al., 1991, J. Neuroscience 11:3888).

Many studies have been performed to examine the role of altered APP expression in AD, but the results have been conflicting (for example, see review article: Unterbeck et al., 1990, Review of Biological Research in Aging, Wiley-Liss, Inc., 4:139).

Studies have also been performed to examine if changes in the relative amounts of the different forms of APP are responsible for amyloid accumulation. The results of such studies have been equally confusing, but have generally supported the conclusion that the relative expression levels of the Kunitz domain containing APP's are elevated in AD (Johnson et al., 1990, Science 248:854). Accordingly, transgenic animals expressing elevated APP 751 have been found to display cortical and hippocampal beta-amyloid reactive deposits (Quon et al., 1991, Nature 352:239).

Recent studies have shown that APP fragments extending from the N-terminus of A4 to the C-terminus of the full length APP molecule (referred to hereinafter as the "C-100 fragment", because it is comprised of approximately 100 amino acids) are also capable of aggregation both in vitro (Dyrks et al., 1988, EMBO J. 7:949), and in transfected cells (Wolf et al., 1990, EMBO J. 9:2079; Maruyama et al., 1990, Nature 347:566). Over-expression of the C-100 fragment in transfected P19 cells has been shown to cause cellular toxicity (Fuck-uchi et al., 1992, Biochem Biophys Res Comm 182:165).

Furthermore, C-terminal fragments containing both the beta-amyloid and the C-terminal domains have been shown to exist in human brain (Estus et al., 1992, Science 255:726), and studies in transfected cell lines suggest that these fragments may be produced in the endosomal-lysozomal pathway (Golde et al., 1992, Science 255:728).

Collectively the above reports suggest that a single proteolytic cleavage of APP at the N-terminus of the A4 region is sufficient to initiate the pathophysiology associated with AD. Recent studies have shown that cultures of primary cells and cell lines (including APP transfectants) secrete 3 to 4 kDa peptides which posses the same N-terminus as beta-amyloid (1-42 amino acids), and could conceivably comprise full length beta-amyloid (Haas et al., 1992, Nature, 349:322; Shoji et al., 1992, Science, 258:126). Such peptides have also been found in the CSF of AD and non AD patients (Seubert at al.,1992, Nature, 359:325, Shoji et al 1992, Id.).

APP is also cleaved at a site within the A4 region in the physiological pathway for secretion of the APP extracellular domain (Esch et al., 1990, Science 248:1122; Wang et al., 1991, J. Biol. Chem. 266:16960). This pathway is operative in several cell lines and necessarily results in the destruction of the A4, amyloidic region of the precursor. Evidence that such a pathway is also operative in the human brain has been obtained (Palmert et al, 1989, Biochem. Biophys. Res. Comm. 165:182).

The enzymes responsible for the normal, non-pathological processing of APP have been termed "secre-tases". C-terminal fragments resulting from secretase action are smaller than the C-100 fragments (defined above) by 17 amino acids, and will hereinafter be referred to as the "physiological C-terminal fragment."

It has been postulated that the net pathological accumulation of A4 is controlled by the relative activities of the pathologic and physiologic pathways of APP degradation.

Thus, several possibilities exist to explain the accumulation of beta amyloid in the brains of persons afflicted with Alzheimer's Disease, as follows:

1) a deficiency in the activity or levels of the secretase(s) involved in the destruction of the amyloidogenic region;

2) altered cellular sorting of APP such that it might become exposed to proteases of the pathologic pathway;

3) an elevation in the levels of the pathologic preoteas(s);

4) a deficiency in the levels of degradative enzymes which otherwise degrade amyloid as fast as it is produced; or

5) an increased susceptibility of APP to pathologic proteolytic degradation caused by mutations in the APP amino acid sequence.

Relatively little is known about the regulation of APP sorting in the cell. A growing hypothesis is that altered

phosphorylation at least in part due to altered protein Kinase C activity causes altered APP trafficking, ultimately leading to changes in APP processing (Buxbaum et al., 1990, Proc Natl Acad Sci USA 87:6003). Thus, treatments designed to alter cellular phosphorylation have caused both qualitative and quantitative changes in the pattern of APP C-terminal fragments.

More Recently, (Nitsch et al., 1992, Science 258:304) it was shown that transfection of cell lines with certain acetylcholine receptor types followed by receptor activation caused an increase in APP processing and secretion, in a process concluded to arise by changes in protein kinase activity. Beside implicating a role for altered phosphorylation, this latter study provides a link between plaque pathology and the established perturbations in cholinergic nerve function charateristic of Alzheimer's Disease.

Despite the above observations, there is currently insufficient knowlege of APP sorting to enable the design of a selective and specific therapeutic agent that could restore balance to any underlying alterations of cellular sorting.

Beta-amyloid must be formed by the direct action of a protease(es). The identification of the so called 'pathologic' brain proteases responsible for the C-100 or beta-amyloid formation is an essential step in an effort to develop therapeutic protease inhibitors designed to block amyloid accumulation. Identification of such enzymes requires the development of specific assays for the activity of such proteases which would allow one to specifically measure the activity of the proteases in the presence of other brain proteolytic enzymes which are present in brain extracts.

Such assays are then used to detect the protease during protease purification. Finally, the assays can be used to measure the effect of potential inhibitors of the enzyme such as is required in pharmaceutical screening for lead therapeutic compounds.

Several studies have undertaken the purification and characterization of both the secretases and purported pathologic proteases. Initial studies utilized assays featuring synthetic peptide substrates that only mimicked the expected cleavage sites within APP. While such assays are useful for measuring the in vitro activity of a purified protease, they rarely possess sufficient specificity to allow detection of one protease in a mixture of proteases such as would be required to monitor a protease purification. Thus, these peptidase assays failed to provide the necessary protease specificity, and the peptidase activities thus quantified were used without success to pursue the purification of candidate APP processing enzyme activities from human brain tissue. Prior to the present disclosure, no credible candidate protease(s) for either process have emerged, and the results of the various studies have been conflicting.

For example, the numerous available studies have proposed that the pathologic protease is: lysosomal in origin (Cataldo et al., 1990, Proc. Natl. Acad. Sci USA 87:3861; Haas et al., 1992, Nature 357:500); a calcium dependent serine protease or a metal dependent cysteine protease (Abrahams et al., 1991, An. N.Y. Acad. Sci. 640:161); Calpain I (Siman et al., 1990, J. Neuroscience 10:2400); a multicatalytic protease (Ishiura et al., 1989, FEBS. Lett. 257:388); a serine protease (Nelson et al., 1990, J. Biol Chem. 265:3836); thrombin (Igarashi et al., 1992, Biochem Biophys Res. Comm. 185:1000); or a zinc metallo-peptidase (WIPO application, W092/07068 by Athena Neurosciences, Inc.).

Similar inconsistencies have arisen in the efforts to identify the secretase, which has been claimed to be: a metallo-peptidase (McDermott et al., 1991, Biochem. Biophys. Res. Comm. 179:1148); an acetylcholinesterase associated protease (Small et al., 1991, Biochemistry 30:10795); Cathepsin B (Tagawa et al., Biochem. Biophys. Res. Comm. 177:377); or a plasma membrane associated protease of broad sub-site specificity (Sisodia, 1992, Proc Nat'l Acad Sci USA 89:6075; Maruyama et al., 1991, Biochem Biophys Res Comm. 179:1670).

The general lack of success of past and current efforts to identify the nature of the APP processing enzymes have stemmed from poor specificity of the assays employed, and from the complex heterogeneity of proteases associated with the cerebral tissue.

The present disclosure describes a method which identifies some of the APP processing enzymes with specific assays based on the proteolytic degradation of recombinant APP in combination with immunochemical detection of the reaction products. The assays of the present invention identify human brain proteases that possess the correct specificity and appropriate localization to play a role in the formation of beta-amyloid from the APP.

The format of the presently disclosed assays in conjunction with the identified proteases afford the capacity to process reasonably large numbers of samples and yields good sensitivity due to the immunochemical method of detection. Furthermore, the simplicity of the assay allows for ready adaptation for routine use by lab technicians and yields consistent, reproducible results. These and other improvements are described hereinbelow.

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SUMMARY OF INVENTION

One goal of the presently disclosed invention is to provide a method for discovering drugs that can be used
to treat Alzheimer's Disease patients. As stated previously, the proteolytic degradation of APP to yield the 39
to 42 amino acid peptide beta-amyloid is the first step in the pathophysiological process of amyloid placque
formation. Several lines of evidence point to a causative role of beta-amyloid and the amyloid placques in the
neuro-degeneration characteristics found in the AD brain. These include:

i) the co-localization of plaque material with degenerating neurons and dystrophic neurites (reviewed in
Price et al., 1989, BioEssays 10:69;

ii) evidence that beta-amyloid can be toxic to neurons in culture (Yankner et al., 1990, Science 250:270);

iii) evidence that beta-amyloid is associated with neuronal degeneration and altered memory when tested
in certain animal models (Flood et al., 1991, PNAS 88:3363; Kowall et al., 1991, PNAS 88:7247); and

iv) co-segregation of certain forms of inherited AD with point mutations in the amyloid precursor protein
(Goate et al., 1991, Nature 349:704; Yoshioka et al., 1991, Biochem Biophys Res Comm 178:1141; Chart-
ier-Harlin et al., 1991, Nature 353:844; Murrell et al., 1991, Science 254:97; Mullan et al., 1992, Nature
Genetics 1:345).

Thus, proteolytic conversion of APP to beta-amyloid appears to be an essential step in the pathogenesis
of AD and, as such, an important target for therapeutic intervention. Identification of the relevant protease ac-
tivities, as well as the development of suitable in vitro screening assays, are therefore essential prerequisites
for the development of therapeutic protease inhibitors that could be used as treatments to block amyloid plac-
que formation in Alzheimer's Disease patients.

The present invention contains two developments which can be used to discover inhibitors of proteolytic
beta-amyloid formation:

1) An in vitro assay comprising a holo-APP substrate and either a highly purified protease that degrades
APP or a crude biological extracts containing unidentified proteases that can degrade APP.

The assay enables the detection of in vitro APP degradation activity to yield C-terminal APP fragments.
When used with crude biological extracts, the assay can be used to monitor the purification of, or to char-
acterize the protease responsible for the detected activity.

Additionally, when used with either a purified protease or a crude biological extract containing un-iden-
tified APP degrading enzyme activities, the assay can be used to measure the inhibition of the APP proc-
essing activity by chemical or biological compounds that are co-incubated in the assay mixture. Inhibitory
compounds thereby identified can have application as therapeutic inhibitors of the in vivo amyloid placque
formation characteristic of Alzheimer's Disease patients.

2) The identification and purification of specific proteases from human brain that can form amyloidic or
pre-amyloidic APP C-terminal fragments when used in conjunction with the in vitro assay system described
in (1), above.

Such enzymes include the aspartic protease, cathepsin D and a chymotryptic-like serine protease
distinct from cathepsin G and inhibited by TPCK and alpha-2 antiplasmin and chymotrypsin inhibitor II from
potato. The identification of cathespsin D is particularly significant. We show that cathepsin D is able to
form C-100 like and beta-amyloid like fragments of 10.0 kDa and 5.6 kDA size, respectively.

This discovery enables the use of any purified or isolated cathepsin D to perform a search for inhibitors
of its activity using either the in vitro assay described in (1), above or simpler high throughput peptidase
assays such as those described in the present invention.

Furthermore, since much is known about the specificity of cathepsin D as well as the design of spe-
cific aspartic protease inhibitors, identification of cathespsin D as an amyloidogenic protease enables both
the development of specific cathespsin D inhibitors using established methods, as well as the utilization of
established cathepsin D inhibitors.

Also shown below is that cathepsin D, unexpectedly, hydrolyzes APP at the peptide bond between
Glu-Val (preferred specificity of cathepsin D is, ordinarily, between hydrophobic residues). This informa-
tion can be used further in the design of cathepsin D inhibitors.

As mentioned above, inhibitory compounds thereby identified have application as therapeutic inhib-
itors of the in vivo amyloid placque formation characteristic of Alzheimer's Disease patients.

APP degrading enzymes identified by use of the present invention can be purified and used to:

i) develop immunochemical reagents necessary to further correlate the co-localization of protease with
AD brain pathology; and

ii) isolate the corresponding protease cDNA. The cloned cDNA can then be used to construct transgenic
animal models for AD in which the effect of protease overexpression can be assessed.

Assays incorporating synthetic peptide substrates are useful for in vitro enzymological studies of highly

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purified protease preparations, but are generally of insufficient specificity to enable the selective detection of a desired protease activity in crude biologic extracts containing a plethora of proteases. For instance, brain tissue is abundant with a wide and varied range of peptide processing and degrading enzymes, which may explain why efforts to isolate specific brain APP degrading proteases with synthetic peptide substrates have been unsuccessful (see Background section, above).

Accordingly, in Example 3, the present disclosure shows that synthetic peptide assays lead to the identification of several peptidases which are unable to degrade APP to yield C-terminal fragments under the specified assay conditions, and that the pattern of APP degrading proteases does not resemble in any way the corresponding pattern of brain peptidases.

A more definitive approach to this problem is the utilization of holo-APP as a substrate, in conjunction with a method of assessing its specific degradation following incubation with protease containing fractions. To this end, the present disclosure describes such a method, wherein the enzymic degradation of recombinant APP by brain protease fractions is monitored by immunoblot using antibodies to the C-terminal region of APP.

Our assay procedure focuses on the formation of C-terminal fragments from APP of size sufficient to include the full length beta-amyloid peptide (a process requiring endoproteolysis, N-terminal to the A4 region).

Human brain tissue (non-AD control or Alzheimer's) is homogenized and then sub-fractionated into a soluble fraction (hereinafter "S"), a post 15,000 g pellet (hereinafter "P-2"), and a microsomal fraction (hereinafter "M"), using conventional ultra-centrifugation. The membranous M and P-2 fractions are solubilized with a Triton X-100 preparation. The resulting soluble fractions from M and P-2, as well as the S fraction, are then separately subjected to chromatography on a Mono-Q strong anion exchange column which results in separation of different brain proteases.

Using a synthetic peptide that mimicks the amino acid sequence surrounding the N-terminus of beta-amyloid, the peptidase activity of individual mono-Q fractions from the purification of M, soluble and P-2 fractions is assessed. Contiguous pools of column fractions are made based on the recovery of discrete peaks of peptidase activity.

The pools of peptidase activity are used to establish assay conditions for the detection of proteolytic degradation of highly pure recombinant amyloid precursor protein purified from a transfected CHO cell line. An immunoblot assay is developed in which antibodies directed either to the APP c-terminal domain or the beta-amyloid region are used to locate c-terminal APP fragments. The assay is used to identify six potentially different proteolytic activities capable of forming APP c-terminal fragments of a sizes large enough to potentially contain full length beta-amyloid. The recovery of APP degrading activity amongst the mono-Q pools is not found to correlate well with the peptidase activity profiles established in step 2. Inhibitor studies reveal that the APP degrading activities include both serine and aspartic protease activities.

The use of the peptidase assay for monitoring enzyme purification is abandoned. Larger supplies of recombinant APP are obtained by expression in a baculovirus directed insect cell system, enabling use of the APP degradation assay as the primary method to monitor APP degrading activity during protein purification. A major aspartic protease activity is identified in fractions from the mono-Q purification of the P-2 fraction.

Further purification and characterisation experiments demonstrate that the enzyme is cathespin D. The cathepsin D is shown to hydrolyse holo-APP forming a beta-amyloid like fragment of 5.6 kDa.

Aprotinin sepharose affinity chromatography is used to attempt to isolate aprotinin sensitive APP degrading activities identified above. A chymotrypsin like serine protease activity is partially purified that can degrade APP to form specific C-terminal fragments of 11, 14 and 18 kDa, that are shown by immunochemical means to contain full length beta-amyloid.

Through this procedure, we have identified several brain protease activities that play, with high probability, a role in amyloidogenic degradation of APP. Each of the identified or unidentified activities described herein can in conjunction with the APP degradation assay be used to screen for selective protease inhibitors of therapeutic value.

As used herein, "APP substrate" shall mean full length APP, whether derived by isolation or purification from a biological source or by expression of a cloned gene encoding APP or its analogs, and fragments of any such protein, including fragments obtained by digestion of the protein or a portion thereof, fragments obtained by expression of a gene coding for a portion of the APP protein, and synthetic peptides having amino acid sequences corresponding to a portion of the APP protein.

APP substrates for the assays of the present invention can be provided as a test reagent in a variety of forms. Although preferably derived from, or corresponding at least in part with the amino acid sequence of, APP 695, derivatives or analogs of other APP isoforms (supra) are contemplated for use in the present method as well. APP 695 can be obtained by biochemical isolation or purification from natural sources such as described in Schubert et al., 1989, Proc. Natl. Acad. Sci. 86:2066; or by expression of recombinant DNA clones encoding the protein or a functional portion thereof (Knops et al., 1991, J. Bio. Chem. 266:7285; Bhasin et al.,

1991, Proc. Natl. Acad. Sci. 88:10307).

The fragments of the APP protein will comprise a sequence of amino acids sufficient for recognition and cleavage by the pertinent proteolytic test sample activity (supra). Isolation of APP from biological material usu-
5   ally will involve purification by conventional techniques such as chromatography, particularly affinity chroma-tography. Purified APP or fragments thereof can be used to prepare monoclonal or polyclonal antibodies which can then be used in affinity purification according to conventional procedures. Resulting purified APP material can be further processed, e.g., fragmented, by chemical or enzymatic digestion. Useful fragments will be iden-tified by screening for desired susceptibility to the pertinent proteolytic test sample activity (supra).
10   As previously stated, the APP substrate can also be prepared by expression of recombinant DNA clones coding for APP or a portion thereof. The cloned APP gene may itself be natural or synthetic, with the natural gene obtainable for cDNA or genomic libraries using degenerate probes based on known amino acid sequences (Kang et al., 1987, Nature 325:733). Other techniques for obtaining suitable recombinant DNA clones, as well as methods for expressing the cloned gene, will be evident to the worker in the field.
15   A variety of convenient methods are applicable to the detection of proteolytic cleavage of the APP substrate in the presence of the test sample. Several of the presently more preferred methods are described below, how-ever, it will be recognized by the skilled worker in the field that many other methods can be applied to this step without departing from the inventive features hereof. In general, any method can be used for this purpose which is capable of detecting the occurrence of proteolytic cleavage of the APP substrate. Such can be afforded
20   by appropriate design of the APP substrate such that cleavage produces a signal producing species, e.g., an optically responsive product such as a colored or fluorescent dye.

Another principal approach involves the sensitive detection of one or more cleavage products such as by immunoassay. Presently, such cleavage product is preferentially a C-terminal fragment of the APP substrate; however, any fragment which appears upon incubation with samples can be the object of detection.
25   The detection of one or more cleavage products characteristic of the pathologic proteolytic activity can be accomplished in many ways. One such method, which is further exemplified in the examples which follow, involves the procedure commonly known as Western blot. Typically, after the incubation of APP with test sam-ple, gel electrophoresis is performed to separate the components resulting in the reaction mixture. The sepa-rated protein components are then transferred to a solid matrix such as a nitrocellulose membrane.
30   An antibody specific to a fragment characteristic of APP degradation is then reacted with the components fixed to the membrane and detected by addition of a secondary enzyme-labeled antibody conjugate. The lo-cation of the resulting bound conjugate is developed with a chromogenic substrate for the enzyme label.

A variety of immunoassay formats which are amenable to currently available test systems can also be ap-plied to the detection of APP fragments. Typically, the APP substrate will be incubated with the test sample
35   and resulting intact APP rendered immobilized (such as by capture onto a solid phase), or alternatively, the test sample is incubated with an immobilized form of the APP substrate. Proteolytic cleavage is then detected by reacting the immobilized APP substrate with an antibody reagent directed to a portion of the APP substrate which is cleaved from the APP substrate or which defines the cleavage site.

The antibody reagent can comprise whole antibody or an antibody fragment comprising an antigen com-
40   bining site such as Fab or Fab', and can be of the monoclonal or polyclonal type. The detection of antibody reagent bound to the immobilized phase is indicative of the absence of the characteristic proteolytic cleavage. Conversely, loss of antibody binding to the immobilized phase is indicative of APP cleavage. The detection of binding of the antibody reagent will generally involve use of a labeled form of such antibody reagent or use of a second, or anti-(antibody), antibody which is labeled.
45   Capture or immobilization of APP can be accomplished in many ways. An antibody can be generated spe-cific to an epitope of APP which is not on the cleavable fragment. Such an antibody can be immobilized and used to capture or immobilize intact APP. Alternatively, a ligand or hapten can be covalently attached to APP and a corresponding immobilized receptor or antibody can be used to capture or immobilize APP. A typical lig-and:receptor pair useful for this purpose is biotin:avidin. Examples of haptens useful for this purpose are flu-
50   orescein and digitoxigenin.

The solid phase on which the APP substrate is immobilized or captured can be composed of a variety of materials including microtiter plate wells, test tubes, strips, beads, particles, and the like. A particularly useful solid phase is magnetic or paramagnetic particles. Such particles can be derivatized to contain chemically ac-tive groups that can be coupled to a variety of compounds by simple chemical reactions. The particles can be
55   cleared from suspension by bringing a magnet close to a vessel containing the particles. Thus, the particles can be washed repeatedly without cumbersome centrifugation or filtration, providing the basis for fully auto-mating the assay procedure.

Labels for the primary or secondary antibody reagent can be selected from those well known in the art. Some such labels are fluorescent or chemiluminescent labels, radioisotopes, and, more preferably, enzymes

for this purpose are alkaline phosphatase, peroxidase, and β-galactosidase. These enzymes are stable under a variety of conditions, have a high catalytic turnover rate, and can be detected using simple chromogenic substrates.

Proteolytic cleavage of the APP substrate can also be detected by chromatographic techniques which will separate and then detect the APP fragments. High pressure liquid chromatography (HPLC) is particularly useful in this regard. In applying this technique, a fluorescently tagged APP substrate is prepared. After incubation with the test sample, the reaction mixture is applied to the chromatographic column and the differential rate of migration of fluorescent fragments versus intact APP is observed.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a-1f show two dimensional contour plots of peptidase activities of control compared to AD human cortex subfractions.

Subfractions were prepared according to Example 1, by ion-exchange (mono-Q) separation of P-2, S and M fractions. Enzymatic cleavage of N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1) by Mono-Q fractions was performed as described in Example 3. Each plot shows the relative amounts of each flourescent product (abscissa) obtained by incubation of each mono-Q fraction (ordinate) under the same incubation conditions. The amount of product is represented vertically by contour lines. Greater numbers of contour lines indicate greater amounts of a particular product. Mono-Q fractions from control S (a), AD S (b), control M (c), AD M (d), control P-2 (e), AD P-2 (f), were subjected to analysis. The roman numerals on the right hand ordinate of the three AD plots locate pooled regions described in Example 3, and which were then assayed according to Example 8, and found to contain significant APP degrading activity.

Figures 2a-2f depict immunoblot analysis of the APP 695 degrading activity associated with selected Mono-Q pools from the ion-exchange separation of M, S or P-2 fractions derived from AD cortex.

The pools were made based on their content of peptidase activity as described in Example 3. Immunoblot assays were performed as described in Example 8. Representative assays for the following pools are shown:

Figure 2a: Activity associated with P-2 pool V:

APP was present in each of lanes 2 to 6. C-100 from PMTI 73 (lane 1), no P2-V blank (lane 2), P2-V (lane 3), P2-V plus EDTA (lane 4), P2-V plus methanol (lane 5), P2-V plus pepstatin A in methanol (lane 6).

Figure 2b: Activity associated with M pool III:

APP was present in lanes 2 to 7.
C-100 from PMTI 73 (lane 1), M-III plus cystatin C (lane 2), M-III plus aprotinin (lane 3), M-III plus captopril (lane 4), M-III plus EGTA (lane 5), M-III plus N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1) (lane 6), M-III without inhibitor (lane 7), prestained molecular weight markers (lane 8).

Figure 2c: Activity associated with S pool I:

APP was present in each of lanes 2 through 6. S-I plus N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1) (lane 2), S-I plus EGTA (lane 3), S-I plus captopril (lane 4), S-I plus aprotinin (lane 5), S-I plus cystatin C (lane 6), C-100 from PMTI 73 (lane 7). Lane 1 contains prestained molecular weight markers.

Figure 2d: Activity recovered in individual mono-Q fractions from the separation of AD P-2:

Mono-Q fractions 38 to 43 corresponding to the conductance region in which P-2 pool VII is otherwise observed were individually examined for APP degrading activity. For each fraction, the incubation was carried out both in the absence (-) or presence (+) of recombinant APP 695. The fraction numbers are located on Figure 2. The C-100 standard used was from PMTI 100. Mr indicates molecular size markers.

Figure 2e: Comparison of the position of migration of C-100 products dir cted either by PMTI 73 or PMTI 100:

5   The protein product of PMTI 73 (lanes 1 and 3) and PMTI 100 (lanes 2 and 4) are shown in comparison with molecular markers (lane 5).

Figure 2f: Typical time course of product formation:

10   APP plus M-III were analyzed at time t= 0 h (lane 1), 5 h (lane 2), 20.5 h (lane 3), 44.5 h (lane 4), and 55 h (lane 5). Molecular weight markers (lane 6), C-100 PMTI 73 (lane 7), and APP without M-III (lane 8), are also shown.

    For each of Figures 2a through 2f, above, migration was from top to bottom. In 2a-2d and 2f, the upper solid arrow locates the position of migration of holo-APP, and the lower solid arrow locates the position of mi-
15 gration of C-100. In Figure 2d, the open arrows locate the positions of migration of putative oligomers of the enzymically generated C-100 fragment. The concentrations of all inhibitors are listed in Table 4, below.

Figures 3a and 3b depict results from further purification of P-2 pool VII by gel filtration.

20   **Figure 3a:** P2-pool VII fractions from Mono-Q 10/10 chromatography were pooled, concentrated to 0.25 ml and applied to a tandem arrangement of two Superose 6HR 10/30 columns equilibrated in 10mM tris HCl buffer pH 7.5 containing 150mM NaCl. Elution was performed at a flow rate of 0.3 ml/min, and column eluent was monitored at 280 nm. Fractions (0.24 ml) were collected and subjected to both peptidase activity, and APP degradation assay using the immunoblot. The arrows locate the region of the chromatogram in which the APP
25 degrading activity was recovered. Also shown are the peptidase activities associated with both K-M (closed circles) and M-D (open circles) bond cleavage. Chromatography was performed at 22°C.

    Figure 3b: The migration of the APP degrading activity relative to the indicated standard proteins of known molecular weight was used to calculate an Mr apparent of the APP degrading protease which is listed in Example 8.

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Figure 4 shows Peptide Epitope Mapping of Murine Monoclonal Antibody C286.8A Raised Against the Beta-amyloid Peptide.

    Micro-titre plates were coated with 50 ng of synthetic APP 695 (597-638) (beta-amyloid 1-42), blocked, then
35 incubated with 100 ul of C286.8A (80 ng of IgG) which had been preincubated (60 min at room temperature) in the presence or absence of the indicated concentrations of competitor peptide. Note, that the peptide 1-7 refers to peptide SEQ ID NO: 1. Following incubation for 60 min at room temperature, plates were washed, and the amount of bound antibody determined by development with horseradish peroxidase-coupled goat-anti mouse polyclonal antibody according to standard procedures (Wunderlich et al., 1992, J. of Immunol. Methods
40 147:1). Percent competition (% C) of antibody binding to the plate was calculated from the absorbance at 450 nm data using the following equation:

$$\% \, C \; = \; 1.0 \; - \; \frac{\text{O.D. (+ competitor) } - \text{ O.D. blank}}{\text{O.D. (- competitor) } - \text{ O.D. blank}} \times 100$$

45   **Figure 5: APP695 processing activity recovered in ion-exchange fractions from the purification of human brain P-2 subfraction.**

    A total of 123 fractions were collected from the column. The first 32 fractions corresponded to the load and wash phase. The salt gradient started at fraction 33. Screens of fractions 3 to 18 (panel a), 21 to 32 (panel b),
50 33 to 38 (panel c), and 39 through 44 (panel D), are shown. For each fraction, the incubation was performed in both the absence (-) and presence (+) of APP695 substrate. Incubations of APP695 for zero or 24 hr is located where appropriate. Incubations were performed as follows. Baculo-derived holo-APP695 (80 nM) was incubated with 5ul of each column fraction in a total of 15 ul containing 100 mM Mes buffer pH 6.5, 0.008 % (v/v) triton X-100, 160 mM NaCl, 6.7 mM tris (from the APP stock). Reactions were terminated after 24 h by addition
55 of SDS-PAGE sample buffer. Immunoblots were developed using the C-terminal polyclonal antiserum of Example 6, as described in Example 8. The arrows locate the product fragments. Fractions 45 to 86 were also tested but showed relatively little activity (therefore not shown). Peak A and B locate the major activities.

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**Figure 6 shows results of purification of P-2 derived APP degrading activity n g l filtrati n: c rrela-
ti n with the elution of cathepsin D.**

5 Panel (a) A 280 nm elution profile for the purification of P-2 peak B on a superose 6HR column. Panels (b)
and (c) corresponding APP c-terminal processing activity in the eluted fractions 49 to 60, determined essen-
tially as described in Example 5. Arrows locate major product bands. Panels (d) and (e), Immunoblot analysis
of eluted fractions using a rabbit polyclonal antibody to cathepsin D (1/300, dilution). The arrows locate the
position of migration of immunoreactive bands. Human liver cathepsin D was also analyzed for comparison.

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**Figure 7 depicts protease inhibitor specificity of protease activities isolated from the P-2 subfrac-
tion.**

Reactions (32 ul) were initiated at 37°C by APP addition to achieve the following initial component conditions:
15 P-2 enzyme (2.54 ug/ml) fraction from the 15-25 kDa region of gel filtration (Figure 6); APP (168 nM), in 96
mM Mes buffer pH 6.5. Reactions were terminated after 26 hr by addition of 15 ul of 3X sample buffer, and
subject to immunoblot analysis using a 1/1000 dilution of the rabbit polyclonal antiserum to the APP C-terminus.
The effect of addition of the following inhibitors is shown: No inhibitor (lanes 7 and 20), 1 mM EDTA (lane 5);
400 uM PMSF in ethanol (lane 9); ethanol alone (lane 11); 100 uM E-64 (lane 13); 10 ug/ml aprotinin (lane
20 22); 100 uM pepstatin A in DMSO (lane 26); DMSO only (lane 24). The effect of incubation of APP for 0hr (lanes
2 and 30) and 26 hr (lanes 3 and 28) are also shown. Lanes 6, 10, 14, 17, 21 and 25 contained enzyme alone.
Lanes 4, 8, 12, 19, 23, and 17 contained the C-100 standard. Prestained molecular weight markers are present
in lanes 1 and 29. The 18 and 28 kDa C-terminal product fragments are located with arrows.

25 **Figure 8 shows time course of Cathepsin D catalysed APP cleavage monitored using an antibody to
the APP c-terminal domain.**

Panel (a) shows time course of APP proteolysis by cathepsin D in the absence (lanes 10-14) or presence (lanes
4-8) of 86 uM pepstatin A. APP was also incubated alone (lanes 1-3). The numbers indicate the time (hr) after
30 initiation of reactions. Reactions were initiated at 37°C by the addition of APP to achieve the following initial
component concentrations: APP (82 nM), cathepsin D (9.2 ug/ml),in 89 Mes buffer pH 6.5. Samples without
pepstatin received the same amount of solvent (1.3 % v/v methanol). At t=0, 43, 84, 140 and 215 minutes ali-
quots (15 ul) were removed mixed with 7.5 ul of SDS-PAGE sample buffer and subject to immunoblot analysis
using the Rabbit antiserum to the APP C-terminal domain. The 18 and 28 kDa reaction products are located
35 with arrows.

**Figure 9 depicts pH and ionic strength dependence of APP C-terminal processing by the P-2 derived
enzyme or cathepsin D.**

40 Panel (a) shows pH dependence observed with cathepsin D and the P-2 enzyme (peak B, Figure 5 following
gel filtration chromatography, Figure 6). Panel (b) is ionic strength dependence for both enzymes at pH 6.5.
Reactions were initiated at 37°C by enzyme addition to achieve the following initial component concentrations:
cathepsin D (9.2 ug/ml) or P-2 enzyme from figure 6 (11.7 ug/ml), 100mM in each of sodium acetate, Mes, and
Tris-HCl, and purified APP (79 nM). At t=0 and 3 hr, aliquots (15 ul) were removed mixed with 7.5 ul of 3X sample
45 buffer and subject to immunoblot analysis with the C-terminal polyclonal antiserum according to Example 8.
The 28, 18 and 14 kDa reaction products are located with arrows.

**Figure 10 shows cleavage of N-Dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID
NO: 7) by cathepsin D and the P-2 enzyme (peak B, Figure 5) following further purification on Super-
50 ose 6HR.**

Reactions (30 ul) were initiated at 37 C by enzyme/inhibitor addition thereby achieving the following t=0 com-
ponent concentrations: Cathepsin D (2.8 ug/ml), or P-2 enzyme from figure 6 (17.5 ug/ml), N-Dansyl-peptide
(24 uM), captopril (0.3 mM) in a cocktail buffer comprising 130 mM in each of acetate, Mes and Tris pH 5.0.
55 Samples contained either pepstatin A (213 uM in 3% v/v final methanol) or an equivalent final concentration
of methanol only. At 0, 2, 4, 8 and 24hr, reactions were terminated by addition of 12 % (v/v) TFA (10 ul) and
subject to HPLC analysis according to example 2 and 3. Representative traces are shown for: P-2 enzyme,
t=0 hr (panel A); P-2 enzyme, t=24 hr (panel B); P-2 enzyme plus pepstatin A, t=24 hr (panel C); cathepsin D,
t=0 hr (panel D); cathepsin D t=24 hr (panel E); cathepsin D plus pepstatin A, 24 hr (panel F).

Figure 11 depicts pH dependence of N-Dansyl-Ile-Ser-Glu-Val-Lys- Met-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID NO: 7) cleavage by cathepsin D and the P-2 enzyme (peak B).

5   Reaction conditions were essentially as described in Figure 10, except that the cocktail buffer was adjusted to the indicated pH values. (a) cleavage by cathepsin D, and (b) cleavage by P-2 enzyme. Rates of cleavage at the -Glu-Val- (closed circles) and -Met-Asp- (open circles) bonds are shown in each case.

Figure 12 is SDS-PAGE analysis of reaction products from the preparative digestion of APP by cath-
10   **epsin D.**

Panel (a) is a photograph of the coomassie stained electroblot prior to excision of bands, panel (b) is the corresponding blot after band excision, and panel (c) is the corresponding immunoblot analysis (1/100 dilution of monoclonal 286.8A) of a parallel series of reactions to those depicted in panels (a) and (b). Reactions in (a)
15   were initiated at 37°C by substrate addition thereby achieving the following initial component concentrations: APP (15.6 uM), cathepsin D (0.17 uM, 7.145 ug/ml), in 40 mM sodium acetate pH 5.0, containing 30 mM NaCl. At t=16 hr the reaction mixture was concentrated to 15 ul by speed vac, mixed with 7.5 ul of 3X sample buffer and subject to SDS-PAGE. Reactions in (c) were performed in essentially the same manner except that the APP concentration was decreased to 3.2 uM. For both experiments (in a and c), incubations were performed
20   with the complete incubation system (lanes 3), and in the absence of cathepsin D (lane 4, cathepsin D added back immediately after addition of the sample buffer). Lane 5 in each case contained cathepsin D only controls, while lane 6 contain a purified APP as migration standard. Prestained molecular weight markers are in lane 1. In (c), the main immunoreactive products are located with arrows.

25   **Figure 13 shows a time course of cathepsin D catalysed APP degradation monitored using a mono-clonal antibody to the N-terminus of beta-amyloid.**

Reactions were initiated at 37°C by APP addition thereby achieving the following initial component concentrations: APP (448 nM), cathepsin D (30 nM), and when included pepstatin A (97.2 μm) in 83 mM sodium acetate
30   buffer pH 5.0. At the indicated time points, aliquots (20 ul), were removed mixed with 10 ul of 3X SDSPAGE sample buffer and subjected to immunoblot analysis using monoclonal antibody 286.8A (1/100). Reactions were performed in the absence (-) or presence (+) of pepstatin A (delivered in methanol). All samples received 2.7 % (v/v) methanol. Lanes 1 and 12 contained prestained Mr markers, Lanes 10 and 18 contained C-100.Mr marker. Lanes 11 and 13 contained APP incubated without cathepsin D for zero and 21hr respectively. The
35   main product fragments are indicated with arrows.

Figure 14 shows the effect of amino acid substitution on the time course of hydrolysis of synthetic peptides by cathepsin D and the P-2 derived enzyme (peak B).

40   Reactions were initiated at 37 C by substrate addition to achieve the following initial component concentrations: Cathepsin D (2.5 ug/ml) or P-2 peak B enzyme (7.5 ug/ml); N-dansyl-peptide (58 uM), captopril (0.3 mM), with or without pepstatin A (213 uM), sodium chloride (75 mM), in 135 mM buffer in each of tris, Mes and acetate buffer pH 5.0. At various times, aliquots (30 ul) were removed, adjusted to 12.5% (v/v) in TFA and subject to RP-HPLC analysis. Time course of hydrolysis for the following substrate/protease combinations are shown:
45   a) N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID NO: 7) by cathepsin D;
             b) N-dansyl-Ile-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID NO: 3) by cathepsin D;
             c) N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID NO: 7) by the P-2 enzyme (peak B, Figure 5 following gel filtration, Figure 6);
             d) N-dansyl-Ile-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID NO: 3) by the P-2 enzyme (peak
50   B, Figure 5).
     Cleavage at the E-V bond (squares, panel A and C), M-D bond (closed diamonds, A and C), or so as to generate the metabolite at retention time 4.4 min (in B and D) are shown.

Figure 15 shows purification of solubilized P-2 fraction on aprotinin sepharose.
55
Experiment was perfromed according to Example 1. (a) is typical A280 nm elution profile, and (b) is an immunoblot assay (rabbit antiserum to C-terminal domain of APP) for APP processing activity in the luted fractions. The arrows indicate the migration of the main APP degradation products. Note the appearance of breakdown products in fractions 8-13 from acid elution.

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Figure 16 shows purification f P-2 derived aprotinin binding pr t ase on mono-Q.

(a) shows A280 nm elution profile, (b), activity of eluted fractions using a rabbit anti-C-terminal APP antiserum
5   (1/1000 dilution) for detection, and (c), activity of eluted fractions using Mab 286.8A.(1/100 dilution of 1.6 mg/ml
pure IgG) for detection. The three arrows indicate the migration of the 11, 14 and 18 kDa C-terminal APP deg-
radation products.

Figure 17 shows pH and ionic strength dependence of APP degradation catalysed by the pool Y ser-
10   **ine protease.**

Panel (a) shows pH dependence. Reactions were initiated at 37°C to achieve the following t=0 component con-
centrations: Pool Y protease (5 ul of fraction 16 from Figure 16), APP (38 nM), in a cocktail buffer comprising
32 mM in each of acetate, Mes and Tris adjusted to the indicated pH values. Reaction mixtures (16 ul) were
15   terminated after 2 hr by the addition of 7.5 ul of 3X sample buffer. Immunoblots were developed essentially
as described in Example 8, using a rabbit polyclonal antiserum to the APP C-terminal domain. Lanes 1 and
12 contain prestained Mr markers. Lane 9 contained C-100 and Lanes 10 and 11 contain APP incubated for 0
and 3hr respecively at pH 6.5 in the absence of pool Y. Panel (b) shows ionic strength dependence. Reactions
were performed essentially as described in (a) except the buffer was 95 mM Mes pH 6.5 containing the indi-
20   cated molar concentrations of sodium chloride. APP cleavage in the absence (lanes 2 to 7) or presence (lanes
9 to 14) of pool Y are shown for each concentration of sodium chloride. Lane 1 and 8 contain Mr markers and
C-100 standard respectively. The arrows indicate the migration of the 11, 14 and 18 kDa APP fragments.

Figure 18 depicts inhibitor selectivity of the pool Y protease.
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Reactions were initiated at 37°C by enzyme addition to achieve the following initial component concentrations
in a 16 ul volume: pool Y # 3-5 (14 ug/ml) after purification on a superdex 75 column, APP (35 nM), in 30 mM
Mes buffer pH 6.5. Reactions were terminated by addition of 7.5 ul of 3X sample buffer. Immunoblots were
developed using the rabbit antiserum to the APP C-terminal domain according to example 8. Data are shown
30   for the effect of the following inhibitors: Panel (a) 860 uM PMSF in methanol (lane 4), 400 uM pepstatin in me-
thanol (lane 6), 5 mM benzamidine (lane 8), 350 uM E-64 (lane 9), 7.7 mM EDTA (lane 10), 15 uM aprotinin
(lane 11), and 0.1 % (w/v) deoxycholate (lane 15). The following controls were also run: no inhibitor (lane 12),
ethanol (lane 3), methanol (lane 5), pool Y only (lane 2), APP only at time zero (lane 13) and 4 hr (lane 14).
Lanes 1 and 7 respectively show prestained Mr markers and the C-100 standard. Panel (b) 1.8 uM alpha-1-
35   antichymotrypsin (lane 2), 156 uM TLCK (lane 3), 46 uM chymotrypsin inhibitor I (lane 4), 119 uM chymotrypsin
inhibitor II (lane 5), 4 uM alpha-2-antiplasmin (lane 6), 51 uM alpha-1-antitrypsin (lane 7), 98 uM chymostatin
administered in DMSO (lane 10), 153 uM methanolic TPCK (lane 12). Controls included: no inhibitor (lane 8),
DMSO (lane 11), and methanol (lane 13). Pre-stained molecular weight markers and C-100 standards were
applied to lanes 1 and 9 respectively. Incubation times of 4 and 2 hr were used in panels a) and b) respectively.
40   The arrows indicate the migration of the 11, 14 and 18 kDa APP degradation products.

DETAILED DESCRIPTION OF THE INVENTION

Example 1. Human Brain Protease Isolation.
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Two general approaches were taken for protease isolation. In the initial studies brain protease activities
were isolated as described under "Method 1", below. Characterization of the resultant enzyme activities ob-
tained from ion-exchange chromatography is described in Examples 3 and 8 and lead to the identification of
six different activities which were able to degrade recombinant CHO cell derived APP (Example 8) but were
50   relatively innactive as peptidases (Example 3).
One of the six activities was subsequently identified as cathepsin D (Example 9), and was further char-
acterised according to its chromatography on gel filtration.
In an alternate approach to attempt to affinity purify some of the human brain serine proteases described
in Example 8 (Table 4), "Method 2" was implemented. This procedure was based on the affinity purification
55   of serine proteases using aprotinin sepharose at an early step, and lead to the identification of a serine pro-
tease(es) (Example 10), which also exhibited the capacity for APP C-terminal processing.

Method 1:

i) **Sub-c llular fractionation.** Sections of human frontal cortex pole 9 region (4.5g) from four separate, age-matched Alzheimer's patients were weighed out while frozen (-70°C), added to 150 ml of 0.32M sucrose at 4°C and scissor minced. The suspension was homogenized in batches using a 100 ml Elvehjem glass teflon potter (10 return strokes). The combined homogenate was centrifuged (1000 g x 10 min) in a Sorval SS-34 rotor.

The loose pellet was removed, re-homogenized and centrifuged as described above. The supernatant for each extraction was combined and centrifuged at 15,000 g x 30 min in the Sorval SS-34 rotor. The resultant "P-2" pellet was resuspended in 100 ml of ice cold 0.32M sucrose by vortexing and stored at -70°C. The supernatant from the last spin was centrifuged at 105,000 g x 60 min to yield the supernatant or soluble fraction ("S"), and the microsomal fraction ("M") which was resuspended in 60 ml of 0.32 M sucrose. Both S and M were stored at -70°C.

Table 1

Summary of protein recoveries:			
Fraction	Volume (ml)	Control (mg)	AD (mg)
Soluble (S)	250.0	315.0	472.0
P-2 pellet	100.0	436.0	412.0
Microsomal (M)	60.0	105.0	89.4

ii) **Solubilization.** The membranous control or AD subfractions (P-2 or M) were solubilized by adjusting to the following conditions: 2% (v/v) Triton X-100 containing 50 mM Tris HCl buffer, pH 7.5. After stirring at 4°C for 3.5 hrs, the suspensions were centrifuged at 105,000 g x 60 min in a Beckman 70 Ti rotor. The following final protein concentrations were used in solubilization, for P-2 (3.9 to 4.0 mg/ml); and for M (1.4 to 1.6 mg/ml). Solubilized supernatants were stored at -70°C for later use. The soluble fraction was not treated with detergent but rather was adjusted to 50 mM in Tris HCl, pH 7.5, by the addition of stock 1M buffer.

iii) **Ion-exchange chromatography.** Chromatography was performed using a Gilson gradient liquid chromatograph (model 305 and 306 pumps) equipped with a 50 ml Rheodyne stainless steel loop injector model 7125, and connected to a Mono Q HR 10/10 column (Pharmacia, Piscataway, NJ). Absorbance of column effluent was monitored at 280 nm using a Pharmacia UV-M detector and a Kippen-zonen chart recorder.

Protein fractions of P-2, microsomal (M), or soluble (S) were loaded onto the column and equilibrated with 50 mM Tris HCl, pH 7.5 (conductivity 1.8 mU at 4°C) at a flow rate of 2 ml/min. The column was then washed with the equilibration buffer until the A280 nm in the eluent decreased to zero whereupon the column flow rate was increased to 4 ml/min.

Proteins were eluted as follows:

Solvents: A = 50 mM Tris HC1 pH 7.5

              B = 50 mM Tris HC1 pH 7.5, containing 1M NaCl.

Program: 0 - 50 % B over 70 min

              hold 50 % B for 10 min

              50 - 100 % B over 10 min

              hold 100 % B for 10 min

              re-equilibrate.

Four mililiter fractions were collected throughout chromatography.

The following protein loads were applied per column run:

P-2, 97 mg (control) and 95 mg (AD);

S, 50mg (control) and 68 mg (AD); and

M, 36 mg (control) and 31 mg (AD).

In the initial studies, eluted fractions were monitored for A280 nm, total protein (Bradford assay), and peptidase activity (as decsribed in Examples 2 and 3). Pools made on the basis of peptidase activity were then prepared (Example 3) and then tested for their capacity to process CHO cell derived APP C-terminally (described in Example 8).

In all further studies however eluted fractions were also individually tested for their capacity for C-

terminal processing of recombinant APP derived by baculo virus directed expression (Example 9).

iv) Gel filtration chromatography. A typical example of gel filtration is depicted in Figure 3. More generally, chromatography was performed as described below. Mono-Q fractions from the purification of P-2, and containing APP C-terminal processing activity were pooled, concentrated to less than 0.25 ml and applied to a tandem arrangement of two Superose 6HR columns equilibrated with 10 mM Tris HCl buffer pH 7.5 and containing 150 mM NaCl. A flow rate of 0.3 ml/min was used througout. Fractions were monitored for A 280 nm, total protein (Bradford assay), peptidase activity, and activity for C-terminal processing of recombinant APP.

Method 2:

i) Sub-cellular fractionation. This was performed essentially as described in Method 1, above.

ii) Solubilization. This was performed essentially as described in Method 1, above.

iii) Aprotinin-sepharose chromatography. Soluble (230 mg), P-2 (216 mg) or microsomal fraction (47 mg) described above was applied to a column of aprotinin sepharose (Sigma. catalog # 42268, 1.5 X 10 cm), previously equilibrated with 20 mM Tris HCl buffer pH 7.0. Once loaded the column was washed with equilibration buffer (100 ml), and then eluted with 60 ml of 50 mM sodium acetate buffer pH 5.0 containing 500 mM sodium chloride. The flow rate was 1.0 ml/min throughout. Eluted fractions (4 ml) were monitored at 280 nm, analysed using the Bradford protein assay, and examined for APP C-terminal processing activity as described in Example 8, using recombinant APP derived by expression in a baculo virus system. Active fractions were capable of forming 11, 14 and 18 kDa (approx.) fragments which were detectable on immunoblots using an anti-APP C-terminal antibodies (see Method 6 for method of antibody production).

iv) Ion-exchange chromatography. Active fractions from the purification of the P-2 fraction on aprotinin-sepharose were pooled, dialyzed against 50 mM Tris-HCl pH 7.5 and applied to a mono-Q column (HR 5/5), previously equilibrated with dialysis buffer. Once loaded, the column was eluted essentially as described in Method 1. above. Active fractions (2 ml), were monitored for A 280 nm, total protein (Bradford assay), and for their capacity for baculo virus derived APP C-terminal processing. A broad peak of APP-degrading activity was observed, which was capable of forming 11, 14, and 18 kDa APP C-terminal fragments which reacted with both the APP C-terminal antibody as well as a monoclonal antibody directed to the N-terminus of the beta-amyloid peptide (see Example 6 for antibody production).

Based on the A 280 nm profile across the region containing active fractions, three pools of activity were prepared each overlapping with a distinctive A 280 mn peak. The pools comprised the following conductance ranges: pool X (12.2 to 14.4 mmho), pool Y (14.9 to 18.9 mmho) and pool Z (20.2 to 22.9 mmho).

v) Gel filtration chromatography. Pools X, Y, and Z were each concentrated to either 2 ml (pool X and Y) or 3.6 ml (pool z) and separately applied to a Superdex 75 column (Pharmacia, Piscataway NJ.) previously equilibrated with 50 mM Tris-HCl pH 7.5, containing 150 mM sodium chloride. Once loaded, the column was eluted with equilibration buffer. Chromatography was performed at a flow rate of 1.0 ml/min throughout. Fractions (1 ml) were monitored for A 280 nm, total protein (Bradford assay) and for C-terminal processing of baculo expressed APP. The gel filtration was calibrated by chromatography of each of the following standard proteins: Thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17.5 kDa) and vitamin B 12 (1.35 kDa).

Example 2. Peptidase assay development.

A peptidase assay was developed to enable the high throughput detection of endoproteases in human brain tissues which might possess a specificity appropriate for APP hydrolysis at the junction between the "extracellular" domain(s) and the N-terminus of the beta-amyloid peptide region. The technology selected utilized dansylated peptide substrates, in conjunction with subsequent detection of fluorescent peptide products by RP-HPLC separation, and post column fluorescent detection.

Evolution of peptide substrate sequence: A fluorescently labelled dodeca-peptide substrate containing the same amino acid sequence as observed surrounding the N-terminal region of the beta-amyloid peptide sequence of human APP was prepared by solid phase peptide synthesis. Peptides were synthesized on an Applied Biosystems model 430A peptide synthesizer using Fmoc / NMP-HoBt chemistry (Fields et al., 1990, Int. J. Peptide Protein Res., 35:161; Knorr et al., 1989, Tetrahedron Letters, 30:1927). Usually, the peptides were cleaved and deprotected in 90% trifluoroacetic acid, 4% thioanisole, 2% ethanedithiol, and 4% liquefied phenol for 2 h at room temperature.

However the peptide: N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His (SEQ ID NO: 2) was found to undergo unwanted carboxy peptidase digestion when incubated with crude tissue fractions. To attenu-

ate carboxy peptidase digestion, the following, modified substrates were designed: N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg -NH₂ (SEQ ID NO: 7) and N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1).

5   This latter peptide was relatively insensitive to carboxy peptidase digestion even in the presence of crude tissue fractions and was used in the peptidase profiling studies of Example 3. The C-terminal alpha amide substrate (SEQ ID NO: 7) was used in the peptidase studies of Example 9 using more purified enzyme fractions. The degradation of either of the peptides was monitored using the HPLC protocol of Example 3, below.

10   **Example 3. Determination of peptidase activities in subfractions of normal-control and AD brains.**

    i) Incubations. Aliquots (20 ul) of column fractions described in Example 1 were incubated with 10ul of a reaction mixture so as to achieve the following final component concentrations: N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1) (50uM), captopril (300uM), in a

15   cocktail buffer comprising 100 mM in each of: MES, Tris, and acetate, pH 6.5.

    Incubation with ion-exchange fractions was performed at 37°C for 24 hrs, after which reactions were terminated by adjusting to 3% (v/v) final in TFA.

    ii) HPLC quantification of proteolytic products. HPLC analysis was performed using a Hewlet-Packard HP1090 complete with binary solvent delivery, heated column compartment, and auto injector. Fluores-

20   cence detection (post column) was performed with an in-line Gilson model 121 filter fluorometer (excitation at 310-410 nm, emission at 480-520 nm) in conjunction with an HPLC chem-station (DOS series) and suitable software for data analysis.

    Aliquots (usually 10 ul) of the above acidified incubation mixtures were injected onto a Hypersil 5uM C18 column (100 x 4.6 mm) fitted with a guard C18 5 uM guard (20 x 4.6 mm). Isocratic separation was

25   achieved using 100 mM sodium acetate buffer, pH 6.5, containing 27% (v/v) acetonitrile. Identification and quantification of resolved metabolites was made possible by comparison with the migration of synthetic peptide products. The structure of which were confirmed by PTC-amino acid analysis and FAB-MS (See Table 2, below).

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Table 2

HPLC retention times of known synthetic peptide standards:

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Peptide (N-dansyl-)	HPLC retention time (minutes)	Cleavage Site
ISEVKMDAEFRHDDDD	2.228 + 0.024	Substrate
ISEVKM	5.398 + 0.019	M-D
ISEVK	3.413 + 0.004	K-M
ISEV	2.692 + 0.003	V-K
ISE	2.135 + 0.002	E-V
IS	4.142 + 0.019	S-E

50   Where: A = Ala, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, M = Met, R = Arg, S = Ser, and V = Val. Also note, the retention time of certain metabolites listed in Table 2, above, differ to those quoted in Example 2 for cleavage of

55   N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His (SEQ ID

NO: 2) due to variables in the HPLC set up. For example, the studies which reflect data listed in Table 2 have relatively longer retention times because chromatography was performed using a guard column in line with the HPLC column.

In all experiments the HPLC column was calibrated for day to day variation in the retention times of the enzymically generated products by analysis of synthetic product standards in parallel with the experimental samples. Data for the proteolytic metabolite profile of individual ion-exchange fractions was collected using the HP CHEM station data acquisition software.

The area under the curve for each of the six cleavage products and their retention times are stored in a peak table file. All peak tables are collated and transferred to a (6 x n) area array in EXCEL (using a custom utility written in programming language C) where n = the number of Mono Q fractions. Each row of the array represents a single peptidase analysis from a Mono Q fraction. Zeros are inserted between each column of data to artificially establish a gradient of values in the row direction.

SpyGlass takes this array and transforms it into a three-dimensional surface in which Mono Q fraction number, cleavage site and Area % for the product formed are the three axes. Contours are defined according to the following criteria set manually within the SpyGlass Program: the first contour line connects contiguous regions of the plot where 1.5% substrate conversion to the particular product was observed. Similarly successive contour lines connecting regions of 5%, 10%, 20%, 30%, 40% and 50%, substrate conversion were also displayed. The resulting contour plots represent brain peptidase maps in which fraction numbers span the ordinate, and peptide bond cleavage sites are on the abscissa, and the amount of product formed is represented by the contours.

Results of peptidase profiling of control and AD brain subfractions.

Figure 1 shows a comparison of the peptidase profiles obtained for the cleavage of the N-dansyl peptide substrate by both control and AD P-2, S, and M fractions subjected to further subfractionation by ion-exchange chromatography. The analysis enables the identification of a high number of potentially different peptidase activities throughout the subfractions of control and AD brain.

For each analysis (a to f in Figure 1), the amount of activity for cleavage at each peptide bond decreased through the series: V-K > K-M > M-D > S-E > E-V, however the K-M and M-D cleavages are of greatest interest because of their greater likelihood of representing the site of APP hydrolysis leading to C-100 formation. The metabolite recovered at 1.6 min is probably due to methionine oxidation to the sulfoxide in the substrate, as treatment of substrate with hydrogen peroxide generates a product of the same retention time (rt). The metabolites formed with rt at 1.0 and 1.3 and 3.3 min are unidentified at present.

For the K-M cleavages, overall levels of activity both in terms of the spectrum of enzymes and peak activity both for control and AD descended through the series P-2 > S = M. This was also true for the M-D cleavages in AD, wherease for the control fractions the order was S > P-2 = M.

Regarding the most abundant peptidase peaks found in AD (only those bounded by more than one contour line), the following number of obviously resolved peptidase peaks could be discriminated: **P-2**, three K-M peaks and one M-D peak; **M**, three K-M peaks and two M-D peaks; **S**, no K-M peaks and one M-D peak. Qualitative comparisons between the levels of the more abundant peaks between corresponding control and AD subfractions revealed only one noteable difference. The difference was observed in the microsomal fractions wherein the control M profile contained a single M-D product around fraction 75, wherease in the AD profile the same region clearly contained a doublet.

Because of potential variation between peptidase levels in the normal and disease state populations it is of little point to highlight quantitative differences in peptidase levels between control and AD.

In summary while it cannot be discounted that there may be qualitative or significant quantitative differences between control and AD fractions in the levels of minor peptidase forms, the overall profiles looked quite similar with only one obvious qualitative difference being apparent amongst the more abundant peaks of peptidase activity.

iii) Consolidation of peptidase activities into discrete pools. Based upon the peptidase Activity profiles obtained using the N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp-Asp (SEQ ID NO: 1) substrate, column fractions from the ion-exchange separation of P-2, soluble (S) and microsomal

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(M) fraction were consolidated into contiguous pools (12 pools for M, 13 pools for P-2, and 14 pools for S). Care was taken in each case to pool the same regions of the chromatography profile both for AD and control fractions (even if no protease was detectable in the corresponding control region), and the precision of the process was checked by monitoring the conductivity of each pool using a YSI model 35 conductivity meter.

Fractions were maintained at 4°C throughout pooling and then stored at -70°C. Each peptidase pool was concentrated using an Amicon Centriprep-10 membrane. Prior to concentrating, each Centriprep membrane was washed in 50 mM Tris/HCl, pH 7.5. A 15 ml Centriprep was used for peptidase pools that contained a volume of 5 ml or more. These pools were concentrated on a Du Pont Sorval tabletop centrifuge at 2700 rpm for approximately 40 min. Pools that contained less than 4 ml were concentrated with a 2 ml Centriprep on a Du-Pont Sorval RC centrifuge (SS34 rotor) at 5000 rpm for 40 min.

After concentrating, each concentrated pool was washed with an equal volume of 50 mM Tris/HCl, pH 7.5. Pools were maintained at 4°C throughout the concentrating process and stored at -70°C. The pools are listed below in Table 3 with the conductivities and the final concentration volumes.

Table 3.

Pooling and concentration of ion-exchange fractions from the separation of human brain S, M and P2 sub-fractions.					
Pool	Conductance	Control		AD	
	(mmho)	(ml)	fold conc	(ml)	fold conc
S I	1.6	0.75	16	0.75	16
S II	1.6	0.75	16	0.75	16
S III	5.0-6.8	1.50	13	0.50	48
S IV	7.1-7.8	0.75	16	0.75	16
S V	8.0-8.4	0.60	13	0.60	13
S VI	8.6-8.8	1.00	16	1.00	8
S VII	9.2-10.8	1.25	10	1.00	24
S VIII	11.1-11.6	1.00	16	0.80	15
S IX	12.8-13.3	1.50	13	1.25	10
S X	15.1-15.8	1.00	12	1.25	13
S XI	20.2	0.75	5	0.75	5
S XII	26.1	0.75	5	1.00	4
S XIII	33.1	0.75	5	0.80	5
S XIV	38.4	0.75	5	0.60	7
P2 I	1.6	13.00	4	6.00	9
P2 II	1.6	1.00	24	1.00	28
P2 III	1.6	1.00	36	0.60	53
P2 IV	1.6	1.00	46	2.00	24
P2 V	1.6	1.00	32	0.50	64
P2 VI	1.6-1.9	1.00	20	0.50	40

P2 VII	2.5-5.2	1.00	56	0.50	72
P2 VIII	5.5-7.7	1.00	16	0.60	53
P2 IX	8.0-8.6	0.50	16	0.50	24
P2 X	9.1-10.0	1.00	16	0.80	20
P2 XI	10.1-13.9	6.00	10	17.00	4
P2 XII	14.1-17.0	6.00	9	8.00	6
P2 XIII	17.1-20.8	1.00	64	11.50	5
M I	1.6	2.00	10	1.00	28
M II	1.8	0.60	25	0.80	15
M III	1.8	0.50	8	0.40	10
M IV	1.8	0.50	8	0.50	8
M V	9.7-10.2	0.80	5	0.50	24
M VI	10.5	0.50	8	0.50	8
M VII	10.9-14.5	1.50	40	1.00	56
M VIII	15.2-15.8	0.80	19	0.80	15
M IX	16.1-16.5	0.80	15	0.80	15
M X	20.7	0.50	8	0.50	8
M XI	34.5	0.50	8	0.50	8
M XII	37.7	0.50	10	0.50	8

Example 4. Expression of recombinant APP 695.

This example describes the method for expressing holo-APP 695 which was then purified as described in Example 7 and then used as the recombinant substrate for the APP degradation assay described in Example's 8 through 10. Two approaches were used.

Initially, a CHO cell expression system was used to generate APP 695. Experiments using this source of APP as a substrate included the inititial activity measurements which lead to the identification of six different protease activities detectable in contigous pools of mono-Q fractions from the purification of human brain P-2, soluble and microsoaml fractions. These studies are described in Example 8.

Subsequently, recombinant APP 695 was obtained by expression using a baculovirus directed system. The greater amounts of APP 695 thereby generated made it feesible to perform the more detailed studies outlined in Examples 9 and 10, leading to the identification of certain APP degrading enzymes.

Both methods of expression are decribed below:

Method 1: Development of a Chinese Hamster Ovary (CHO) cell line expressing holo-APP 695.

a) **Vector construction.** A known 2.36 Kb NruI/SpeI fragment of APP 695 cDNA from FC-4 (Kang et al., supra) was filled in by the large fragment of E. coli DNA polymerase I and blunt-end inserted into the SmaI cloning site of KS Bluescript M13+ (Stratagene Cloning Systems, La Jolla, CA.) creating pMTI-5 (App 695 under the T3 promoter). A new optimal Kozak consensus DNA sequence was then created using site-specific mutagenesis (Kunkel et al., 1987, Methods in Enzymology, 154:367-382) with the oligo: 5'-CTCTAGAACTAGTGGGTCGACACGATGCTGCCCGGTTTG-3' (SEQ ID NO: 8) to create PMTI-39. This

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plasmid was next altered by site specific mutagenesis (Kunkel et al., Id.) to change the valine at position 614 to a glutamate (open reading frame numbering according to Kang et al., Id.).

The full length APP cDNA containing the optimal Kozak consensus sequence and Val to Glu mutation was then cut out of PMTI-39 with NotI and a HindIII partial digest. The 2.36 Kb APP 695 fragment was then gel purified and ligated into NotI/HindIII cut pcDNAINeo (Invitrogen Corp. San Diego, CA.) to create PMTI 90 in which the APP 695 expression is placed under the control of the CMV promoter. The Val to Glu mutation was sequence confirmed and the vector used to stably transform CHO cells.

b) Generation of stable CHO cell lines expressing APP 695 mutenes.

Chinese Hamster Ovary K-1 cells (ATCC CCL 61) were used for transfection with the APP 695 construct. Twenty micrograms of an expression plasmid containing APP 695 and a neomycin drug resistance marker was transfected into 1×10^7 CHO cells in 0.5 ml PBS by electroporation using a Bio-Rad Gene Apparatus (Bio-Rad Laboratories, Richmond, CA.). A single pulse of 1200 V at 25 μf capacitance was administered to the cells.

Following electroporation, cells were incubated in ice for 10 minutes and collected by centrifugation. The cell pellet was resuspended in Alpha MEM, 10% fetal calf serum at a density of 5×10^4 cells/ml, and 1 ml aliquots were distributed into each well of five 24-well tissue culture cluster plates. After 48 hours incubation, cells containing the neomycin drug resistance marker were selected by addition of 1 ml of media containing 1 mg/ml Geneticin (GIBCO-BRL, Grand Island, NY.) and incubation was continued and bi-weekly changes of drug containing media.

Drug resistant cells were tested for APP 695 expression by Western blotting. Cells positive for APP 695 expression were cloned by limiting dilution, and individual clones were isolated and tested for APP 695 expression. A clone positive for APP 695 expression was subcultured and expanded into roller bottles for large scale production of APP 695 expressing cells and subsequent isolation of recombinant protein.

Method 2: Expression of Recombinant Holo-APP 695 using Baculovirus Infected Insect Cells.

a) Construction of Recombinant Vector. The Baculovirus vector pVL1392 (Invitrogen) was cut Xba I (in the polylinker) and ligated with the gel isolated 2.36 Kb Xba I fragment from pMTI-39 (APP695 NruI/SpeI into KS Bluescript M13+ SmaI site, T3 orientation, with a new Kozak and XbaI site at the SmaI/NruI blunt fusion site). This created pMTI-103, which was transformed into DH5α, selected on Amp, and a lithium prep of the plasmid DNA made for transfection.

b) Cells and Virus. Spodoptera frugiperda (Sf9) cells, purchased from the American Type Culture Collection (ATCC) were grown as suspension cultures at 28 C. in TNMFH media (Summers, M.D. and Smith, G.E. (1987) A manual of Methods for Baculovirus Vectors and Insect Cell Procedures. (Bulletin no. 1555, Texas Agric. Exp. Stn. and Texas A & M Univ., University Station, TX) containing 10% fetal calf serum. Wild type AcMNPV DNA was purchased from Invitrogen, San Diego, CA.

c) DNA Transfection and Plaque Assays. Foreign DNA was inserted into the genome of AcMNPV at the polyhedrin gene locus by homologous recombination by cotransfection of purified plasmid DNA (4ug) and linear viral DNA (1ug) into Sf9 cells using the calcium phosphate procedure (Summers et. al (1987) Supra). Viruses which were released by the transfected cells were purified by 2 rounds of plaque assay (Summers et. al. (1987), Supra.), where recombinant viruses were identified by visually screening for polyhedrin-negative plaques. Purified recombinant viral cultures were Subsequently screened for their ability to produce APP in infected cells by western blot analysis.

d) Recombinant Protein Production. 5 liter batches of Sf9 cells, grown as suspension cultures in TMNFH media containing 10% fetal calf serum at 1×10^6 cells/ml, were infected with recombinant virus at a M.O.I of 1. Cells were harvested 24 hours post infection and cell lysates prepared for purification of recombinant protein.

Example 5. Development of expression vectors for the production of recombinant C-100 standard by transient infection of mammalian cells.

The C-100 peptide fragment contains the C-terminal portion of APP which spans from the N-terminus of the A4 peptide to the C-terminus of full length APP (see above, BACKGROUND section). The C-100 fragment is the purported initial degradation product leading to the ultimate formation of the A4 peptide.

In one embodiment of the present invention, cell lysates from Hela S3 cells (ATCC CCL 2.2) expressing recombinant C-100 are analyzed in the immunoblot assay in parallel with the recombinant APP samples that have been incubated with brain fractions, subfractionated by Mono-Q chromatography (See Example 3). The migration and detection of the C-100 fragments serves both as a size marker for the genesis of products

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formed by pathologic proteases as well as a positive control for the immunodetection of C-terminal APP fragments in general.

Comparison of the size of enzymically generated products with the size of the C-100 fragment can provide insights into whether or not the enzymically generated fragments result from cleavage close to the N-terminus of the A4 peptide or alternatively within the A4 segment as would be catalyzed by secretase.

a) Plasmid construction

Two methods were used to make plasmids for C-100 expression. Each plasmid shall be identified separately as either PMTI 73 or PMTI 100.

PMTI 73 Construction: The commercially available plasmid PUC-19 was digested with EcorI to eliminate its polylinkers. Commercially available PWE16 was then inserted into the digested PUC-19 to create PMTI 2300. PMTI 2301 was derived from PMTI 2300 following BamHI/Hind III digestion using an oligonucleotide adapter. The EcoRI promoter fragment of APP was inserted into the HindIII site of pMTI 2301 by blunt end-ligation to produce PMTI 2307.

PMTI 2311 was generated by ligating the BamHI fragment from FC-4 (Kang et al., supra) into the BamHI site of PMTI 2307. The XhoI fragment from FC-4 was inserted into the XhoI site of PMTI 2311 to generate PMTI 2312. PMTI 2323 was generated by insertion of the 2.2 kb BglII/EcoRI fragment from the EcoRI genomic clone of the mouse metallothionein-I gene into the ClaI site of PMTI 2312. To generate minigene PMTI 2337, the sequences between the KpnI and BglII sites of PMTI 2323 were deleted and the clone was ligated using synthetic oligonucleotide adaptor, sp-spacer-A4.

PMTI 2337 was cut with Bam H1/SpeI and the fragment ligated into the Bam H1/Xba1 restriction sites of bluescript KS (+) (Stratagene) to create PMTI 2371. PMTI 2371 was cut Hind III/NotI to release a 0.7 kb fragment coding for the terminal 100 amino acids of APP 695. Also encoded was the sequence for signal peptide. This insert was ligated into the Hind III/NotI site of pcDNAINEO (Invitrogen Corp.) to create the plasmid PMTI 73.

PMTI 100 Construction: PMTI 90 (see Example I) was cut XbaI/HindIII to release a 0.6 kb fragment again coding for the terminal 100 amino acids of APP 695 and this was ligated to the XbaI/HindIII site of pcDNAINEO to create PMTI 100. In each case vectors,inserts and plasmids were purified by methods known to those skilled in the art.

b) Transfection and expression of C-100 Fragment. Preparation for small scale expression of C-100 standard was initiated by seeding 5×10^5 cell Hela S1 cells in each well of a 6 well costar cluster (3.5cm diameter) 24 hours before use.

Sufficient vaccinia virus vTF7-3 was trypsin treated to infect at a multiplicity of 20 plaque forming units per cell, mixing an equal volume of crude virus stock and 0.25 mg/ml trypsin, then vortexed vigorously. The trypsin treated virus was incubated at 37°C for 30 minutes, with vortexing at 10 minute intervals. Where clumps persisted, the incubation mixture was chilled to 0°C and sonicated for 30 seconds in a Sonicating water bath. The chilled sonication was repeated until no more clumps were detected.

The trypsin treated virus was then diluted with sufficient serum free DMEM for each well with Hela S1 cells to have 0.5 ml of virus. Medium was aspirated way, then the cells were infected with virus for 30 minutes, with rocking at 10 minutes intervals to distribute the virus.

Approximately 5 minutes before infection was ceased, fresh transfection mixture was prepared as follows: To each well was added 0.015 ml lipofection reagent (Betheseda Research Labs, Gathersburg, MD.) to 1 ml OPTIMEM (Betheseda Research Labs, Gathersburg, MD.) in a polystyrene tube, mixing gently. Vortex was avoided. Then, 3 μg CsC1 purified DNA was added and mixed gently.

Virus mixture was aspirated from cells, then the transfection solution was introduced. The resulting mixture was incubated for three hours at 37°C. Each well was then overlaid with 1 ml of OPTIMUM and incubated at 37°C in a CO_2 incubator overnight.

Cells were harvested at 20 hours post transfection by centrifugation, and lysates were prepared on ice with the addition of 0.2ml of a lysis buffer which contained 1% Triton X-100, 10 μg/ml BPTI, 10 μg/ml Leupeptin, 200 mM Nacl. 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM EDTA, adjusted to pH 7.5. Complete lysis was monitored by light microscopy, and harvested immediately. Lysis took less than 1 minute to complete, with delay at this step causing lysis of nuclei resulting in a gelatinous mess.

Recombinant lysates were stored at -20°C for later use. Preferably, recombinant lysates should be diluted 1:50, in (3X) SDS PAGE sample buffer which is devoid of 2-mercapto ethanol prior to freezing.

A comparison of the size of the proteins produced by expression with either PMIT 73 or PMTI 100 using SDS-PAGE/ immunoblot with the APP C-terminal antibody was performed (Figure 2e). The study showed that PMTI 100 directed the expression of a single immunoreactive band, wherease, PMTI 73 directed the expression of two major bands of similar molecular size. A less intense band of intermediate. size was also evident in PMTI 73 when applied to gels in higher amounts (Figures 2a-2d).

Application of the PMTI 100 protein to SDS PAGE gels in higher amounts results in the appearance of a series of fainter bands (eg. Figure 2d). Besides an intense band of C-100 monomer of apparent Mr 11.7 kDa, fainter bands are observed at Mr 25.5 kDa, 35 kDa and 45 kDa, which are attributed to the formation of dimeric,
5 trimeric and tetrameric aggregates, respectively, of the C-100 monomer. An additional faint band of Mr 18.9 kDa is also observed. Similar phenomena have been reported in the literature with similar interpretations (Dyrks et al., 1988, EMBO J. 7:949).

The largest of the three bands produced by PMTI 73 was slightly larger than the single band observed with PMTI 100. Amino acid sequence analysis of the largest band from PMTI 73 expression showed that the
10 signal peptide sequence was cleaved from the initial translation product to yield a C-100 fragment containing 5 extra amino acids at the N-terminus.

Example 6. Production of immunochemical reagents.

15 Three different immunochemical reagents were used in the studies of the present invention:

i) A Rabbit polyclonal antiserum which recognized the C-terminus of APP was obtained and used for immunoblot detection of C-terminal APP fragments generated by proteolytic processing according to the assay conditions described in Example 8;

ii) An affinity purified antibody which recognized the C-terminus of APP was prepared and used to syn-
20 thesis an immunoaffinity column for the affinity purification of APP expressed in a baculo virus directed system (see Example 7); and

iii) a mouse monoclonal antibody which recognizes the N-terminus of the beta-amyloid peptide was generated and used in an immunoblot assay to determine whether C-terminal APP fragments generated by proteolytic digestion of holo-APP 695 contained the full length beta-amyloid peptide (see Examples 9, and
25 10 for specific applications).

The method of generation of each of the three immunochemicals is presented below.

i) Rabbit polyclonal antiserum to the C-terminus of APP. Antisera were elicited to the C-terminal domain of human APP 695, and were prepared in accordance with the method as described in Buxbaum et al., 1990, Proc. Nat'l. Acad. Sci. 87:6003-6006. A synthetic peptide (hereinafter "β APP 645-694") corre-
30 sponding to the COOH-terminal region of APP 695 was obtained from the Yale University, Protein and Nucleic Acid Chemistry Facility, New Haven, CT.

β APP 645-694 was used to immunize rabbits to elicit polyclonal antibodies. Sera were screened by immunoblot analysis of lysates of E. coli that expressed a fusion protein including the amino acids 19 through 695 of human APP 695. Sera which were immunoreactive against the recombinant fusion protein
35 were further screened for immunoprecipitating activity against [^{35}S] methionine-labeled APP 695, which was produced from B APP 695 cDNA by successive in vitro transcription (kit purchased from Stratagene, La Jolla, CA) and translation (reticulocyte lysate kit purchased from Promega Corp., Madison, WI).

ii) Polyclonal antibody affinity column for the purification of holo-APP.

Purification of Synthetic APP C-terminal Peptide Immunogen. 80-90 mg of crude synthetic peptide (P-142)
40 spanning the C-terminus of APP(649-695) with a Cysteine residue at the N-terminus was purified by HPLC (yield 42%;34mg).Amino acid analysis, N-terminal sequence analysis and Laser Desorption Time of Flight Mass Spectrometry showed the purified peptide to be a mixture of full length and N-terminally truncated peptides (2/1 full length to truncated).

Immunization of Rabbits with Purified P-142 immunogen. The HPLC purified peptide APP(649-695) was
45 used to immunize rabbits. Two rabbits each received an initial challenge with 125 ug of peptide in complete Freunds Adjuvant followed by subsequent boosts of the same amount of peptide in incomplete Freunds Adjuvant at three week intervals. Fourteen bleeds were collected over a 9 month interval and optimal production of Ab was observed for bleeds at 16 thru 32 weeks (shown by western analysis with Vacinnia C100 and CHO APP). Bleeds in this interval were pooled for an approximate volume of 90-100mls of anti-sera.
50 Preparation of an immobilized APP649-695 affinity matrix for purification of antisera. 9.7 mg of purified peptide APP(649-695) was coupled to maleimide activated BSA using the Pierce Imject activated Immunogen Conjugation kit with BSA. About 40% of the Peptide (3.88mg) was coupled to BSA as determined by Ellman's Reagent. The BSA coupled peptide was separated from uncoupled peptide by gel filtration (Purification buffer from kit= 83mM NaH2PO4 pH 7.2;900mM NaCL). The pooled void volume from the
55 gelfiltration column (2.9mg P-142 conjugated to BSA/12.5mls) was coupled to 1gm(3.5mls) of CnBr activated sepharose (>90% peptide conjugate coupled by standard Pharmacia protocol). Remaining sites were blocked with Ethanolamine. The sepharose affinity matrix was packed into a 1.0 x 3.5cm glass column.

Purification of Rabbit Polyclonal Antibody using the APP(649-695) affinity column. The combined rabbit

anti-sera from bleeds of optimal Ab production were pooled (100mls/3.9gms protein) and diluted 1:1 (v/v) with wash buffer (100mM NaHCO3 pH8.3;750mM NaCl) and loaded onto the peptide affinity column at 1.0ml/min at 4°C. After loading (200mls), the column was washed with wash buffer (75ml) until A280 returned to zero.The IgG was eluted with 100mM Glycine pH2.5 (40ml). One minute fractions were collected into tubes containing 100ul of 1.0M Tris HCl pH8.0. The neutralized low pH IgG eluant was pooled (35mls;14.7mg) and dialyzed against 1.0 liters of 100mM NaHCO3 pH8.3;500mM NaCl at 4°C.

Preparation of Immunoaffinity column: Coupling purified Rabbit IgG to Sepharose. 5.0 gms of CnBr Sepharose was activated with 50mls of coupling buffer (100mM NaHCO3 pH8.3;500mM NaCl) and mixed with dialyzed IgG pool on an orbitron for 21 hrs at 4°C. After coupling, the resin was rinsed 1X with coupling buffer through a sintered glass filter, followed by 3x rinses with 100 ml ea of blocking buffer.(100mM NaHCO3 pH8.3;500mM NaCL;1.0M Ethanolamine. Two succesive intermediate rinse steps with coupling buffer (100mls), then low pH buffer (100mM NaOac pH 4.0; 500mM NaCl(100ml) and a final rinse with coupling buffer (100ml) completes the resin preparation. The coupling was 87% for a total of 17.5 mls of resin. (0.727mg IgG/ml res-in).

iii) Generation and epitope mapping of a monoclonal antibody to the beta-amyloid peptide.

Hybridoma Methodology. Balb/c mice were immunized by multiple injections of a mixture of the following two synthetic peptides: 1) APP amino acids 597 to 638 of holo-APP695 (numbering according to Kang et al., Id.) containing beta amyloid, and 2) APP695 amino acids 645-695 containing the C-terminal domain. Splenocytes from immunized animals were fused with X63/Ag 8.653 mouse myeloma cells using standard procedures (Herzenberg et al., 1978, In: D.M. Weir (Ed.), Handbook of Experimental Immunology, pp 25.1-25.7, Blackwell Scientific Publications, Oxford, UK). Supernantants from the resultant hybrids were tested for the presence of anti-peptide specific antibodies using an EIA in which the beta amyloid peptide immunogen was bound to the microtitre plate. Cultures secreting antibody which reacted with the synthetic peptide used as immunogen were cloned twice by limiting dilution, and their isotype determined as described (Wunderlich et al., 1992, J. of Immunol. Methods, 147:1). Secreted IgG was purified from the serum free fermentation broth of cloned hybridoma cells by protein-A affinity chromotography of the spent culture fluid.

Epitope Mapping. One of the anti-peptide monoclonal antibodies, an IgG 2b designated C286.8A, gave good reactivity with synthetic beta-amyloid peptide both by EIA as well as by immunoblot assay. The epitope reactivity of the monoclonal was determined using a competitive EIA. Synthetic peptides containing amino acids 597-612, 597-624, 597-638, 608-624, 621-631 and 645-695 of human APP695 (numbering according to Kang et al., Id.) as well as N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1) were tested for the ability to block binding of C286.8A to APP 597-638.

Peptides which are recognized by the antibody will, if preincubated with the antibody in solution, deplete the solution concentration of the antibody available for subsequent reaction with beta-amyloid peptide bound to a microtitre plate. The result of such an experiment is shown in Figure 4 and described herein below.

Only peptides APP 597-612, 597-624, 597-638, and N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1) were able to inhibit C286.8A binding in a dose-dependent fashion. These peptides contain respectively amino acids 1-16, 1-28, 1-42 and 1-7 of the beta-amyloid sequence (numbering from the N-terminal aspartate residue). Peptides devoid of any beta-amyloid sequence such as APP 645-695, or containing the beta-amyloid peptide sequence 12-28 or 25-35 (APP 608-624 and APP 621-631 respectively) did not inhibit binding of the monoclonal antibody to the homologous antigen. These results show that the reactive epitope for this monoclonal antibody resides at least in part, in the first 7 amino acids of the A4 region of human APP, ie, APP 597-601.

Example 7. Purification of recombinant holo-APP 695.

Initial studies of APP C-terminal processing were performed using recombinant APP695 expressed in CHO cells as described in Example 4, above, and purified as described by Method 1, below. Characterization experiments using this substrate are described in Example 8 below and led to the identification of six potentially different APP degrading enzymes capable of C-terminal processing.

Subsequently, a baculovirus expression system was developed (see Example 4), providing higher APP levels than could be achieved with the CHO expression system. The purification of holo-APP695 from the baculo virus system is described in method 2 below. The purified baculo virus derived holo-APP 695 was used to conduct the protease characterisation experiments described in Examples 9. and 10 below.

All steps were performed at 0 to 4°C unless indicated otherwise. Holo-APP 695 was detected by immunoblot analysis using an anti human APP 695 C-terminal antibody essentially as described in Example 8, below.

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Method 1. Purification of holo-APP695 from a Stably Transf cted CHO Cell Line.

a) Isolation of plasma membranes. Whole cell pellets (179 g) from continuous culture of CHO cells in roller bottles (See Example 4) were collected by centrifugation (1500g X 5 min), and resuspended to a total volume of 600 ml in 50 mM tris-HC1 buffer pH 8.0 containing sodium chloride (30 mM), magnesium chloride (1 mM), EDTA (10 mM), PMSF (200 µg/ml), E-64 (42 µg/ml) and pepstatin (3.8 µg/ml). The cells were homogenized using a teflon potter (10 return strokes), then layered (25 ml per centrifuge tube) onto 10 ml of homogenization buffer containing 41% sucrose and devoid of the protease inhibitors EDTA, PMSF, E-64 and pepstatin. Following centrifugation (26,800 RPM X 60 min, in a Beckman SW-28 rotor, the interfacial layer was carefully removed (approximately 150 ml in combined volume), diluted with an equal volume of homogenization buffer (minus protease inhibitors), resuspended with a teflon potter (3 return strokes), and recentrifuged as described above to yield a tightly packed pellet. The supernatant was decanted and the pellet resuspended in 100 ml total volume with 50 mM tris HC1 pH 8.0 (teflon potter 3 return strokes). Re-centrifugation (50,000 RPM X 60 min in a Beckman 70 Ti rotor), yielded a pellet which was resuspended to a total volume of 57 ml in 50 mM tris HC1, pH 8.0.

b) Solubilization of Plasma Membranes. Thirty seven milliliters of the above resuspended CHO plasma membrane preparation were added sequentially to a cocktail of protease inhibitors and stock 20% (v/v) triton X-100 to achieve the following component concentrations: EDTA (1 mM), E-64 (24 µg/ml), PMSF (53 µg/ml), pepstatin A (11 µg/ml), and triton X-100 (2.2% v/v, final), in the homogenization buffer (total solubilization volume of 45 ml) described above. After gently rocking of the mixture at 4°C for 30 min, the non-solubilized material was removed by centrifugation (50,000 RPM X 40 min in a Beckman 70 Ti rotor). The supernatant containing solubilized holo-APP was filtered through a 0.45 µM disc filter.

c) Purification of solubilized holo-APP 695 by strong anion exchange chromatography. The above supernatant containing holo-APP 695 was diluted with an equal volume of distilled water and applied to a Mono-Q RH 10/10 column previously equilibrated with 20 mM tris-HCl buffer pH 8.0 containing 0.1% triton X-100. Once loaded the column was eluted in a linear gradient of 0 to 1M NaC1 contained within a total volume of 210 ml of equilibration buffer. The flow rate was maintained at 3 ml/min throughout. Proteins eluting between a conductivity range of 17 to 22 mmho (4°C) contained the majority of immunoreactive APP 695, and were combined and dialyzed for 4 hours versus 2L of 5 mM tris-HC1 pH 8.0 containing 0,025% triton X-100, and clarified to remove slight turbidity by centrifugation (26,800 x 60 min in a Beckman SW 28 rotor).

d) Heparin agarose chromatography. The clarified sample was applied to a column of heparin agarose (15 x 1.6 cm) previously equilibrated with dialysis buffer. Upon loading a light brown band formed within the top 1/3 of the column. Once loaded, 5 min fractions were collected (a flow rate of 1 ml/min was used throughout). The column was then eluted stepwise with 85 ml of equilibration buffer in which the sodium chloride was successively adjusted to the following final concentrations: 0, 150, 300, 600, and 2000 mM. The majority of the immunodetectable holo-APP eluted at 600 mM NaCl, with the next quantitative fraction being recovered at 300 mM. The APP recovered at 300 mM and 600 mM NaC1 were collected separately and stored in aliquots at -80°C. The APP used in the following studies were from the 300 mM fraction. The yield of partially pure APP from the 300 mM heparin agarose eluent was 5.5 µg (Bradford assay) per gram of wet CHO cell pellet. The APP in the preparations was judged to be about 25% pure based upon SDS PAGE analysis.

Method 2. Purification of Holo-APP695 from Recombinant Baculo Virus Infected Insect Cells.

a) Solubilization of cell pellets. The cell pellets harvested from two 5L fermentation runs were combined (total 8.9 g of detectable protein), added to 160 ml of 0.32 M sucrose containing the following inhibitor: pepstatin A (25 ug/ml); leupeptin (25 ug/ml); chymostatin (25 ug/ml); antipain (25 ug/ml); aprotinin (25 ug/ml), benzamidine (4 mg/ml), PMSF (0.87 mg/ml), and EDTA (25 mM), and homogenized by teflon potter (10 return strokes). The homogenate was centrifuged (105,000g X 1 h in a Beckman 70 Ti rotor) and the pellet was then resuspended by teflon potter (10 return strokes) in 160 ml of 10 mM Tris-HCl buffer pH 7.5 containing 0.5 M NaCl and the same inhibitors and concentrations as listed above. After brief sonication (Branson Sonifier Cell, 2 min power level 4), Triton X-100 was then added to a final concentration of 5 % (v/v), and the suspension was gently stirred for 20 min at 4 C. The mixture was centrifuged (50,000 RPM X 60 min, in a Beckman Ti 70 rotor), and the first supernatant (574 mg of protein) carefully removed for heparin-agarose chromatography. The pellet was resuspended by teflon potter (20 return strokes) in 160 ml of 10 mM tris-HCl buffer pH 7.5 containing 0.5 M NaCl, and each of the inhibitors at the concentrations listed above. Solubilization with 5% (v/v) triton X-100, and subsequent centrifugation was performed as

described above to yield a second solubilized supernatant (683 mg of protein).

b) Radial flow chromatography on heparin-agarose. Both of the supernatants obtained above were purified separately on heparin agarose as follows. The supernatants were diluted by addition of purified water and 1M Tris pH 9.5 to a volume of 3.5 L, a conductance of 1.8 mmho, and a pH of 8.0, and applied to a Superflow 250 column (Sepragen) containing 250 ml of packed resin and previously equilibrated with 5 mM Tris-HCl buffer pH 8.0 containing 0.1 % triton X-100. Once loaded, the column was washed with 3L of equilibration buffer and then eluted with equilibration buffer containing 600 mM NaCl. A flow rate of 30 ml/min was used throughout. Fractions (45 ml were monitored for A 280 nm, total protein (Bradford assay), and the levels of immunoreactive APP deteced by immunoblot against the anti APP C-terminal antiserum of example 6 i). Fractions containing significant APP were combined and subject to antibody affinity chromatography.

c) Antibody affinity chromatography. The 600 mM elution pool from the purification of the first (containing 276 mg of protein) and second supernatant (containing 113 mg of protein) on heparin-agarose were combined, adjusted to pH 8.3, and applied to an antibody affinity column (10.5 X 1.5 cm) comprising affinity purified C-terminal antibody coupled to sepharose as described in example 6 ii), and previously equilibrated with 100 mM sodium bicarbonate buffer pH 8.3 containing 500 mM NaCl, 0.1 % triton X-100. Chromatography was performed at a flow rate of 1 ml/min throughout. Once loaded, the column was washed with 70 ml of equilibration buffer, and then eluted with 50 ml of 100 mM glycine, pH 2.4 containing 0.1% triton X-100. Fractions (5ml) were collected into 0.5 ml each of 1M tris-HCl pH 8.0, and monitored for A280 nm, total protein (Bradford assay), and the presence of immunodetectable APP as above. Fractions containing significant APP were combined. The combined heparin agarose eluent was cycled through the affinity pufication procedure a total of five times. The APP pool recovered from each successive purification was combined for a total of 9 mg of APP.

d) Strong anion exchange chromatography. Combined fractions from antibody affinity chromatography (9.0 mg of protein) were applied to a mono-Q HR 5/5 column previously equilibrated with 20 mM tris-HCl buffer pH 8.0 containing 0.025 % (v/v(triton X-100, and 150 mM NaCl. Once loaded, the column was eluted with a linear 0.15 to 1M NaCl gradient in a total of 70 ml. A flow rate of 0.5 ml/min was used throughout. Eluted fractions containing significant immunodetecable APP were combined and stored in aliqouts at -80 C until used. The final preparation of APP was >95% pure based on SDS-PAGE developed with coomassie brilliant blue stain, exhibited an amino acid composition that was within 86% agreement with the theoretical composition, and exhibited the following N-terminal sequence for the mature protein: Leu-Glu-Val-Pro-Thr-Asp-Gly-Asn-Gly-Leu-. 5.6 mg of purified protein was obtained from the pellet from the two 5 L fermentation runs.

Amino acid analysis was performed essentially as described elsewhere (Dupont, D.R., Keim, P.S., Chui, A.H., Bello, R., Bozzini, M., and Wilson, K.J., "A comprehensive approach to amino acid analysis", in Techniques in Protein Chemistry, ed. by Tony E. Hugli, Academic Press, 284-294 (1989)). Samples were hydrolyzed under argon in the vapor phase using 6N hydrochloric acid with 2.0% phenol at 160°C for 2 h. Phenylthiocarbamoyl-amino acid analysis was performed on an Applied Biosystems model 420A Derivitizer with on-line model 130A Separation System and Nelson Analytical model 2600 Chromatography Software.

Example 8. The immunoblot assay for the detection of the degradation of APP 695 catalyzed by human brain protease subfractions.

a) Incubation with substrate APP

i) 5 ul aliquots of ion-exchange fractions (obtained from steps as described in Example 1) or concentrated pools of fractions (Example 3) are incubated for 24 hrs at 37°C with recombinant human APP 695 (10.75 ul), which was adjusted to 140 mM final in MES buffer pH 6.5 by the addition of the required amount of 2M stock buffer. The final buffer concentration in the incubation was 95 mM, pH 6.5. During the incubation time, proteolytic degradation of some of the APP 695 occurs to yield lower Mr fragments.

ii) The proteolytic reaction was terminated by addition of 7.5 µl, of the following 3X Laemlie SDS-PAGE sample buffer: 1.5 M Tris HC1, pH 8.45, containing 36% (v/v) glycerol and 12% (v/v) SDS, 10% (v/v) 2-mercaptoethanol, and trace bromophenol blue tracking dye. Samples were heated (100°C X 8 min), and then cooled.

b) SDS PAGE analysis:

The reaction mixtures (15 µl) were applied to the wells of a 10 to 20% acrylamide gradient Tricine gel (routinely a 1.0 mm thick, 15 well Novex precast gel, Novex Experimental Technology, San Diego, CA). The gel was run under constant voltage conditions, and at 50 V until the sample enters the gel whereupon the voltage was raised to 100 V. Electrophoresis was discontinued when the tracking dye reaches to within 0.5 cm of the gel bottom. The gels were calibrated using prestained Mr markers ranging in Mr from 3 to

195 kDa (Bethesda Research Laboratories, Gaithersburg, MD.). Ten microlitres each of a kit containing high and low molecular weight markers were mixed with 10 µl of 3X sample buffer, and treated as described in section (a)(ii). The following molecular weight marker proteins were present in the kit as pre-stained markers: Myosin H-chain (196 kDa); phosphorylase B (106 kDa); bovine serum albumin (71 kDa); oval-bumin (45.3 kDa); carbonic anhydrase (29.1 kDa); betalactoglobulin (18.1 kDa); lysozyme (14.4 kDa); bovine trypsin inhibitor (5.8 kDa); and insulin A and B chains (3 kDa).

c) Immunoblotting:

i) The gel was then transferred to a mini trans-blot electrophoresis cell (Biorad Labs, Richmond, CA.). Proteins were electro-blotted onto a ProBlott (TM) membrane (Applied Biosystems, Foster City, CA.), for 1 hour at 100 V (constant), using the following transfer buffer maintained at 4°C:20 mM Tris HC1 buffer pH 8.5 containing 150 mM glycine and 20% (v/v) methanol.

ii) The ProBlott membrane was removed and placed in 15 ml of blocking buffer of the following composition for 1 hour at room temperature: 5% (w/v) non-fat dried milk in 10 mM Tris HC1 buffer pH 8.0 containing 150 mM NaCl.

d) Immunodetection of APP and C-terminal degradation products:

The membrane was transferred to 15 ml of blocking buffer containing a 1:1000 dilution of rabbit poly-clonal antiserum elicited to a synthetic human APP 695 C-terminal peptide immunogen and incubated at 4°C over night.

The membrane was rinsed with three successive 15 ml volumes of blocking buffer with gentle shaking for 5 minutes. The membrane was then transferred to 15 ml of blocking buffer containing a 1:1000 dilution of alkaline phosphatase-coupled Goat anti-Rabbit IgG (Fisher Scientific, Pittsburgh, PA.), and incubated at room temperature for 90 minutes. The membrane was then rinsed with three successive 15 ml volumes of blocking buffer with gentle shaking for 10 minutes.

The membrane was next washed with three consecutive 15 ml volumes of alkaline phosphatase buffer for 5 minutes each, comprising: 100 mM Tris HC1 pH 9.5, containing 100 mM NaC1 and 5 mM MgC1$_2$. The gel was next incubated in the dark with 15 ml of 100 mM Tris HC1 pH 9.5, containing 100 mM NaC1, 5 mM MgC1$_2$ and 50 µl of BCIP substrate (50 mg/ml, Promega, Madison, WI.) and 99 µl of NBT substrate (50 mg/ml, Promega). Incubation was continued until there was no apparent further intensification of low Mr immunoreactive bands (typically 3 hours at room temperature). The gel was then rinsed with deionized water and dried.

Analysis of the capacity of Mono-Q pools of subfractionated human AD cortex to enzymically degrade APP 695 to generate C-terminal fragments.

Each of the P-2, S and M pools described in Example 3 were subject to the immunoblot assay described above. The specificity of the immunologic detection method, in combination with the use of the authentic APP substrate molecule provide a selective method to detect the activity of the APP degrading enzymes in comparatively crude biologic extracts, avoiding the need to use highly purified enzyme preparations. Thus, certain of the partially purified pools possessed a proteolytic activity which was capable of formation of C-terminal APP fragments in a time dependent manner. Representative examples of the immunoblot analysis of human AD brain are shown in Figure 2 for the P-2 V (panel a), M III (panel b) and S I (panel c), as well as for individual fractions prior to pooling of a P-2 VII pool (panel d).

Time course experiments, for example as depicted in Figure 2f, for pool M III showed that these fragments were not present in the substrate or enzyme fractions at time 0. Furthermore, incubation of the substrate alone did not result in their formation (for example see Figure 2a, lane 2, Figure 2d, lane 2, Figure 2f, lane 8). The size range of the bands varied between Mr approximately 11.5 kDa and 25 kDa, depending upon the enzyme fraction, but the number of different products formed in the reactions were surprisingly low. At pH 6.5, eight out of a total of 39 AD pools were found to have such activities. The pools could be distinguished from each other based upon i) brain sub-fraction, ii) ionic strength of column elution, and iii) qualitative APP cleavage pattern.

Six selected pools (designated "M-III, M-VIII, S-I, S-III, P-2 V, and P-2 VII") were found to contain significant APP degrading activity. Corresponding control brain pools also contained some of the above activities, but it was not possible to determine whether the levels of the activities were different or not, between control and AD pools. Each of the above six pools had an enzyme activity capable of forming an 11.5 kDa APP C-terminal fragment.

The proteolytic product of MR 11.5 kDa was of particular interest because in further studies it was usually the major immuno-detectable C-terminal product, and was found to co-migrate with a recombinant C-terminal fragment of APP comprising an open reading frame that would start with the N-terminal aspartate of the beta-

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amyloid peptide and extend to the C-terminus of the full length molecule (the C-100 fragment). This co-migration is exemplified in Figure 2d. The implication of this is that the 11.5 kDa band is the product of endoproteolysis of APP at or near the N-terminus of the A4 region, and that the above protease activities capable of forming this fragment might play a role in vivo, in the genesis of amyloidogenic peptides.

Figure 2d shows that at least in the case of P2 pool VII, the 11.5 kDa C-terminal enzymatic product of APP proteolysis is capable of aggregation. In addition to the appearance of the peptide band at Mr 11.5 kDa which comigrates with the PMTI 100 driven C-100 standard, and the 18 kDa fragment, there appear other bands at Mr 24.3, 27.4 and 35.5 kDa. The 24.3 and 35.5 kDa bands are of a Mr expected for dimers and trimers, respectively, of the C-100 fragment, and roughly comigrate wth the corresponding faint bands in the C-100 which are due to aggregation (see Example 5 for a details).

Figures 2a-2c also help to show that the assay can be used to examine the effect of classical protease inhibitors. For example, it is apparent from Figure 2a, that P-2 V is inhibited partially by methanol and completely by methanolic pepstatin A, while M-III (Figure 2b) and S-I (Figure 2c) are both completely inhibited by aprotinin and cystatin. Thus, the assay, in one embodiment, is applied to the search for novel in vitro inhibitors of the APP degrading enzymes. The potent compounds thereby identified are tested for in vivo efficay using a suitable animal model such as a transgenic animal designed to overexpress APP or a beta-amyloid-containing fragment thereof.

Table 4, below, summarizes some of the properties of the six main pools of APP degrading activity recovered from the Mono-Q fractions, including peptide product sizes, apparent pH dependence for product formation, and the effects of commercially available protease inhibitors.

Table 4

Properties of human AD brain fractions active in APP proteolysis[1].

Pool[2]	Conductance (mmho)	Fragment size[3] (kDa)	Optimum pH	Trial[4]	Inhibitors[5] (pH 6.5)
M III	1.6	11.5	(6.5)	A,B	aprotinin cystatin
		>11.5	(5.0)	–	N.D.
S I	1.6	11.5	(6.5)	A,B	aprotinin cystatin
		>11.5	(6.5–8.0)	A,B	cystatin
P2 V	1.6	11.5	(6.5)	A,B	pepstatin A aprotinin
		18.0	(8.0)	–	N.D.
P2 VII	2.5–5.2	11.5	(6.5–8.0)	A	PMSF
		>11.5	(6.5–8.0)	A	PMSF
		18.0	(6.5–8.0)	A,B	N.I.
S III	5.0–6.8	11.5	(8.0)	A	N.I.
		>11.5	(8.0)	A	N.I.
		18.0	(8.0)	A,B	N.I.
M VIII	15.0–16.0	11.5	(8.0)	A,B	N.I.
		>11.5	(8.0)	A,B	N.I.
		18.0	(8.0)	B	N.I.

[1]Reactions were performed as described in Example 8. using pools prepared and concentrated according to Example 3

[2]Concentrated protease pools as defined in Example 3

[3]Specific C-terminal APP fragments (products) from proteolysis

[4]The inhibitors studied were:

trial A: PMSF (0.8mM), EDTA (7.7mM), Pepstatin A (400μM), E-64(260μM);

trial B: EGTA (1mM), cystatin (20μM), captopril (300μM), aprotinin (15μM), N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Asp-Asp-Asp (SEQ ID NO: 1) (90μM).

[5]Compounds causing complete inhibition are listed.

N.D. = not determined due to low or inconsistent levels of activity; N.I. = no inhibition observed.

Some of the activities possessed pH optima in the alkaline range, and were unlikely to be due to the actions of lysozomal cathepsins. This observation is significant because several investigators have reported that pathologic APP processing is performed by proteases within the endosomal-lysosomal pathway (Cataldo et al., 1990, Proc. Natl. Acad. Sci USA 87:3861; Benowitz et al., 1989, Experimental Neurology 106:237; Cole et al., 1989, Neurochem Res 14:933). Enzymes within this pathway would be expected to exhibit acidic pH optima.

Based on the available data, M-III and S-I are highly similar by all the listed criteria, and probably represent the same enzyme cross contaminating each of the S and M fractions. It is probable, therefore, that human brain contains a minimum of five different protease activities capable of degrading APP to yield a 11.5 kDa C-100-like product fragment.

Table 4 shows that some of the activities were insensitive to inhibition by any of the inhibitors tested, and these enzymes may represent members of an unusual group. The activities involved in the formation of 11.5 kDa C-100 fragments in M-III and S I, are either of serine or cysteine type, or represent members of an unusual group. Alternatively, these fractions may contain both a serine and cysteine protease with both enzymes playing an obligatory (sequential) role in the production of C-100. P2 V contains both an aspartic protease activity and a serine protease activity. P2 VII contains a serine protease activity based upon its sensitivity toward PMSF. However, in subsequent studies pepstatin-inhibitable activity was also noted, indicating the co-localization in P2 of an aspartic protease along with the serine protease activity in Table 4. None of the enzyme activities in S III or M VIII were sensitive to any of the inhibitors tested. In no case was it possible to demonstrate inhibition of APP degradation by co-incubation of the enzyme pool with the N-dansyl peptide substrate used in Example 3.

Comparison of the recovery of APP degrading activities (Example 8) with the peptidase activities of the Mono-Q pools (Example 3 and Figure 1) clearly shows that there is little correlation between the two activities. Thus, the APP degrading activities were largely contained in pools that exhibited comparatively little peptidase activity. This suggests that the APP degrading activities are poor peptidases and may require an intact folded APP substrate for activity, or alternatively (but less likely) the peptides selected represent the wrong locus for pathologic APP processing. Regardless, this finding explains why other investigators have been unsucessfull in identifying common APP degrading enzymes using assays based on peptide substrates.

From the above considerations, it is concluded that the present assay is of a sufficient specificity to enable the isolation of specific APP degrading enzymes from human brain.

In further studies, we have used the immunoblot assay to track the recovery of the P-2 VII associated APPase. Work was focused on this pool because it represented the most abundant of the six characterized activities, and because it generated C-terminal fragments that seemed to be amyloidic (Figure 2d). It represents the major activity recoverable from ionexchange separation of the P-2 subfraction, and is eluted at a point in the gradient which did not coincide with the main peaks of peptidase activity.

The P-2 VII fractions displaying APPase were pooled and subject to size exclusion chromatography on two tandem Superose 12 columns (Pharmacia). Peptidase and APPase activities in the eluted fractions were analyzed (Figure 3a). While the K-M cleavage activity seemed to overlap in part, the peak of M-D activity once again did not coincide with the peak of APPase. Calibration of the chromatography against known molecular weight markers yielded a median Mr apparent of 31.6 kDa with an uncertainty of plus or minus 6.5 kDa for the APPase activity of the P-2 VII fractions (Figure 3b).

Example 9. Identification of Cathepsin D as an APP C-terminal processing enzyme.

Having obtained greater quantities of holo-APP695 by using the baculovirus expression system described in Example 4, Method 2, and purification scheme of Example 7, Method 2, it became possible to track the recovery of APP degrading enzymes in individual column fractions from the purification of human brain enzymes, rather than assess the content of APP degrading enzymes in pools of fractions made on the basis of peptidase activity (as had been done in Example 8.

The content of APP degrading enzyme activity is shown in Figure 5 for individual mono-Q fractions from the purification of solubilized P-2 fraction according to the method of Example 1. When compared with a similar analysis of soluble and microsomal fractions subjected to Mono-Q chromatography, the relative staining intensity for enzymatic C-terminal APP fragments was consistently greatest in the P-2 subfraction from Mono-Q. APP degrading activity in the P-2 was recovered from Mono-Q as two distinct migration peaks (A and B, Figure 5).

Peak A eluted in the loading and low ionic strength wash, i.e. in a region roughly corresponding to the recovery of P-2 V, seen in our initial studies (Table 4), whereas peak B overlapped with the pooled region in which P-2 VII activity was previously observed (Table 4), and shown to comprise both serine and aspartic protease activities. Similar sized degradation products were observed with both the peak A and B activities at Mr approx. 28, 18 and 14 and <11 kDa, although the relative staining intensity of the 18 kDa band was much greater in peak B than in peak A. Peak B was pooled and subject to purification on superose 6HR as described in Example 1, Method 1. Eluted fractions contained two qualitatively distinct types of activity which overlapped in their elution profiles. The activity which produced an APP breakdown pattern most closely resembling that observed with the original peak B fractions (figure 5) was recovered in fractions 51 through 56 from gel filtration (figure

6 b and c), consistent with an apparent Mr of 15 to 25 kDa. This elution peak was preceded by elution of an activity which predominantly formed an 18 kDa breakdown product, and is presumably catalyzed by a protease of larger Mr apparent. This latter activity probably corresponds to the serine protease activity previously de-
5   scribed in the P-2 VII pool in Example 8, Table 4. Active fractions from the gel-filtration purification of peak B (and within the 15-25 kDa Mr region) were tested for inhibition by clasical protease inhibitors (Figure 7). These studies confirmed that peak B activity was largely catalysed by an aspartic protease as determined by quantitative inhibition by Pepstatin A.

Comparatively few human aspartic protease are known. Those that have been identified include, Cathe-
10   psins D and E, Renin, and pepsin. To test the possibility that the activities that we observed might correspond to some of these enzymes, commercial preparations of human Renin (Calbiochem, Sandiego, CA catalog # 553864), and human cathepsin D (human liver, Cal Biochem, San Diego, catalog # 219401) were examined for their capacity to enzymically degrade baculo derived holo-APP.

The commercial preparation of cathepsin D used throughout the studies described herein was electrophor-
15   etically homogeneous on SDS-PAGE developed with silver stain, and exhibited an amino acid composition which showed 93 % agreement with the theoretical value based on the known protein sequence.

Whereas renin was inactive (not shown), cathepsin D selectively cleaved the APP so as to produce a similar pattern of C-terminal degradation products to those observed with P-2 peak B (Figure 5) described above from Mono-Q. Thus, commercial cathepsin D preparations degraded holo-APP in a time dependent fash-
20   ion to produce major C-terminal products of approximate Mr 18 and 28 kDa. Inhibition of the activity by pepstatin A confirmed the involvement of cathepsin D in the reaction (Figure 8).

A commercial polyclonal antibody to human cathepsin D was obtained (Dako Corp, Carpinteria, CA, catalog # A561), and found to be reactive toward human cathepsin D on immunoblots, generating an immunoreactive band of Mr 28 kDa. The antibody was used in an immunoblot assay to examine if chromatography fractions
25   from the mono-Q purification of either P-2, soluble or microsomal fractions contained immunoreactive cathepsin D.

Significant amounts of cathepsin D were observed in Mono-Q fractions of the P-2 and soluble fractions (data not shown) that possessed APP degrading activity. Interestingly, two chromatographically distinct peaks of cathepsin D reactivity were observed in the analysis of P-2 mono Q fractions each of which coincided with
30   peaks A and B (not shown). The immunoreactive aspartic protease, cathepsin D associated with peak A activity coincided with the region in which P2 V of Example 8 had been previously identified. This suggested that peaks A and B could be due to multiple forms of cathepsin D. Multiple forms of cathepsin D have been described elsewhere and attributed to differences in post-translational modification of a single gene product. Immunoblot analysis of gel-filtration, column fractions from the further purification of P-2 peak B (Figure 5) showed the
35   presence of a peak of cathepsin D immunoreactivity exactly co-incident with the peak of APP degrading activity (Figure 6B).

In addition to co-migrating with cathepsin D immunoreactivity and degrading APP similarily to cathepsin D, Peak B protease further purified by gel filtration exhibited the same pH optima (between pH 4-5) and ionic strength dependence as cathepsin D for formation of C-terminal APP degradation products (Figure 9). Finally,
40   the immunoreactive band observed in the P-2 fraction exhibited a similar pI (4-6) to that reported for cathepsin D when subject to preparative IEF on Biorad Miniphor chromatography system (not shown). Collectively, these data strongly support the fact that the pepstatin sensitive APP protease activities observed following mono-Q fractionation of human brain P-2 are due to the action of cathepsin D.

The peptidase activity of the peak B protease (purified on gel filtration) and cathepsin D were then com-
45   pared using the synthetic peptide N-Dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID NO: 7) as the substrate. Both enzymes hydrolysed the peptide in a time dependent fashion albeit at quite low rates. For both enzymes, the major cleavage was observed at the -Glu-Val- bond and to a lesser extent at the -Met-Asp- bond (Figure 10). Note, that in Figure 10, most of the Met-Asp cleavage product was further converted to the Glu-Val product by the 24 hr. time point depicted. As expected, the peptidase reactions catalyzed by
50   Cathepsin D and the P-2 enzyme preparation were both inhibited by pepstatin A.

Both enzymes exhibited acidic optima at pH 4 for the hydrolysis at the -Glu-Val- bond (Figure 11). Hydrolysis at the -Met-Asp- bond also exhibited an acidic optimum with cathepsin D (< pH 3.0), but with the P-2 enzyme, two optima were observed (at pH ,3.0 and pH 7.0), possibly due to participation of an additional contaminating P-2 protease in the reaction with a neutral pH optimum (Figure 11). Cathepsin D usually hydrolyses
55   between hydophobic residues. However at acidic pH values, protonated (neutral) forms of the Asp and Glu side chains might appear sufficiently hydrophobic to satisfy the subsite binding requirements of the protease. The pKa of the Asp side chain is more acidic than the Glu residue, and would be protonated to a lesser degree than the Glu residue throughout the pH range examined in Figure 11. This may explain the lower cleavage rates at the -Met-Asp- bond with cathepsin D, and the hint at a lower pH optimum for cleavage (< pH 3) at this

site when compared with the -Glu-Val-bond.

Further experiments explored the identity of the peptide bonds in APP that are cleaved by Cathepsin D. Larger amounts of APP were subject to cathepsin D hydrolysis at pH 5.0. Limited proteolysis under non-dena-
5 turing conditions was employed. Incubation mixtures were analysed by SDS-PAGE, immunoblotted, and then the individual product bands located either by coomassie staining or by immunodetection with the anti-beta-amyloid monoclonal described in Example 6(iii). The main bands located with coomassie blue were subject to N-terminal sequencing.

Figure 12 shows both a coomassie stained blot as well as an immunoblot (using the anti-beta-amyloid
10 monoclonal antibody) of such a reaction mixture. As a control, incubations were also performed in the absence of cathepsin D (wherein cathepsin D would be added back to the incubation mixture after addition of SDS-PAGE sample buffer), or in the absence of APP 695 substrate. Eight main product bands were observed by coomassie staining (Figure 12a) of the complete incubation mixture, and which were also absent from either of the controls. Some but not all of those bands also reacted with the A4 monoclonal (Figure 12c), which rec-
15 ognises an epitope within the first 5 residues of the beta-amyloid peptide. N-terminal analysis of the coomassie stained products yielded the sequences listed in the table below.

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Table 5.

N-terminal sequences of major proteolytic products following incubation of purified cathepsin D with holo-APP 695.

Proteolytic band # (Fig. 12a)	product size (kDa)	N⁰-terminal sequence	peptide bond hydrolyzed
1	3.9	R–V–I–Y–E–R–M– Q–A–V–P–P–R–P–	–L–R– –L–Q–
2	4.4	Q–A–V–P–P–R–P– R–V–I–Y–E–R–M–	–L–E– –L–R–
3	5.6	V–K–M–D–A–E–F– Q–A–V–P–P–R–P–	–E–V– –L–E–
4	6.3	V–S–D–A–L–L–V–	–F–V–
5	10.0	V–S–D–A–L–L–V– L–E–V–P–T–D–G– V–K–M–D–A–E–F–	–F–V– –A–L– –E–V–
6	15.8	G–A–D–S–V–P–A–	–F–G–
7	24.5	L–E–V–P–T–D–G	–A–L–
8	56.2	L–E–V–P–T–D–G	–A–L–

ᵃ The amino acid sequences were determined with an Applied
Biosystems model 477A Protein Sequencer operated in the gas phase
with on-line model 120A Analyzer and Nelson Analytical model 2600
Chromatography Software.

Where: A = Ala, D = Asp, E = Glu, F = Phe, G = Gly, I = Ile,
K = Lys, L = Leu, M = Met, P = Pro, Q = Gln, R = Arg, S = Ser,
T = Thr, V = Val and Y = Tyr.

With exception of band 7 all sequences were assigned for the first
ten cycles. For band 7, sequencing was discontinued after cycle 6.

As expected several products were observed corresponding to cleavages that were largely consistent with those reported for cathepsin D hydrolysis of other substrates (Moriyama et al., 1980, J. Biochem, 88:619). Exceptions to the reported cathepsin D specificity included the -Glu-Val- cleavage to form the major product of band 3, and the minor product of band 5, as well as the -Leu-Arg- cleavage products of bands 1 and 2 (Table 5).

The cleavage of the -Glu-Val- bond at APP 593-594 is consistent with the observed capacity of the cathepsin D to cleave the corresponding bond in the peptide substrate (as described above) in a pepstatin inhibitable reaction. Cathepsin D usually hydrolyses between pairs of certain hydrophobic residues. Cleavage at the Glu-Val bond, though unexpected, probably occurs under acidic (pH 5) conditions due to protonation of the side chain of the glutamate residue (pKa = 4.25), rendering it neutral.

Indeed, it can be calculated that 18% of the -Glu- side chains should be protonated at pH 5.0. Such acidic conditions occur in lysozomes and secretory granules, or could be induced upon tissue damage, or following hypoxia or local ischaemia.

Most significantly, cathepsin D generated a 5.6 kDa product (band 3, Table 5), by atypical hydrolysis at the -Glu-Val- bond three amino acid residues N-terminal to the purported N-terminal -Asp- residue of the common form of beta-amyloid. The fragment was absent in the equivalent sections of the blot taken from the incubation without cathepsin D. Furthermore, the fragment is of the right size (5.6 kDa) to contain full length beta-amyloid peptide, and its generation suggests that cathepsin D must also cleave the APP at a second site close to the C-terminal region of the beta-amyloid peptide.

In fact, a precursor substrate for such a C-terminal cleavage was also identified in band 5, which exhibited an Mr (10.0 kDa). The size of this fragment suggests that it contains most if not all of the C-terminal domain and that it arose by a single -Glu-Val-cleavage at APP 593-594.

APP 695 contains numerous other peptide bonds that would seem to have been ideal substrates for cathepsin D cleavage yet were not cleaved by cathepsin D. The fact that they were not hydrolyzed reflects the high degree of sequestration of these sites away from acess to cathepsin D within the folded APP structure: most of the hydrophobic pairs would be expected to locate to the hydrophobic APP protein core. The same considerations explain why the sites that were shown to be hyrolysed by cathepsin D (Table 5) did not not always contain the optimal cathepsin D recognition motif. To be located on the protein surface, such sites would have to contain a greater degree of polarity or charge than would be ideal for cathepsin D catalysed cleavage. It is noteworthy in this regard that three of the five internal cleavage sites contained two proline residues each within eight residues of the scissile bond. Such residues are often associated with a brake in secondary structure or with turns which often are found at the protein surface.

In a parallel immunoblot (Figure 12c) several of the product peptides (located with arrows), reacted with the monoclonal antibody C286.8A to the N-terminal residues of beta-amyloid. These included a band at Mr 5.6 which migrated in the same position as band 3 in Figure 12a (Table 5) a doublet between Mr 9 to 10 kDa comigrating with band 5 in Figure 12a (Table 5), and a doublet at Mr 14 kDa, a doublet at 16 to 18 kDa comigrating with band 6, Figure 12a, and a band at Mr 40 kDa. Of the bands sequenced (Table 5), only bands 3, 5 and 6 comigrated with bands detected by immunoblot in Figure 12c. Consistent with this, only these same three bands in Table 5 were of the appropriate N-terminal sequence and size to contain the beta-amyloid epitope.

The time course of formation of the beta-amyloid immunoreactive degradation products described in Figure 12 was performed under slightly different molar ratios of cathepsin D and APP (Figure 13), both in the absence and presence of pepstatin A. In the absence of inhibitor, a time dependent accumulation of low molecular weight

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fragments was observed, starting initially with the formation of a bands at Mr approx. 16-18 and 28 kDa respectively. At 2hr, a band at Mr approximately 40 kDa was observed. While the 16-18 and 40 kDa bands further intensified beyond 2 hr., the intensity of the 28 kDa band remained constant beyond this time point. The intensities of the 16-18 and 40 kDa did not increase further beyond 8 hr. Between 8 hr. and 21 hr. there was a substantial increase in the intensities of detectable bands at Mr approx. 14, 10 and 5.6 kDa. Since these latter three bands did not intensify in parallel with either the 16-18, or 40 kDa, it is probable that the 14, 10 and 5.6 kDa bands were derived from secondary degradation of either or all of the 16-18 or 40 kDa bands. The 16-18, 10 and 5.6 kDa bands described in Figure 13 correspond to the same Mr bands listed in Table 5 and shown in Figure 12c. All of the bands observed in Figure 13 were inhibited by pepstatin A confirming that they arose by the action of cathepsin D.

The implication of cathepsin D as a major protease in amyloidosis of Alzheimer's Disease now explains other observations made concerning the disease. Firstly, there is growing evidence that APP accumulates in lysozomes, and is processed there to yield amyloid bearing fragments (Haas et al., 1992, Nature 357:500). Amyloid deposition is favored at the acid pH of the lysosome (Burdick et al., 1992, J. Biol. Chem. 267:546). Secondly, while cathepsin D is a lysozomal protease, it has also been shown by histochemistry to be present in significant levels associated with amyloid deposits in Alzheimer's brain (Cataldo et al., 1990, Proc. Natl. Acad. Sci USA 87:3861).

Thirdly, beta-amyloid released by cells in culture comprises a minor N-terminal sequence starting at residue Val 594 (Haas et al., 1992, Nature 359:322) which is three amino acids N-terminal to the more abundant sequence beginning at the Asp 597 residue commonly seem in beta-amyloid 1-42. The minor sequence probably arises by direct endoproteolysis at the -Glu-Val- bond at position 593-594, ie the same site as shown presently to undergoe specific proteolysis by cathepsin D. It is emphasised here that since cathepsin D can hydrolyse both the -Glu-Val- and -Met-Asp- bonds, it has the necessary specificty to form both of the beta-amyloid fragments sequenced by Haas et al.

Fourthly, the cysteine preoteases inhibitors E-64 and leupeptin were without effect on the release of beta-amyloid by LC-99 cells while general lysozomal inhibitors blocked the release (Shoji et al., 1992, Science, 258:126), showing that beta-amyloid formation by these cells was catalysed by a lysozomal enzyme other than a cysteine protease. A remaining candidate protease for such a reaction would be lysozomal cathepsin D which is not inhibited by the cysteine protease inhibitors used in their studies.

Finally, APP contains a stretch of hydrophobic residues between the C-terminus of beta-amyloid and the membrane anchore sequence. Some of the peptide bonds in this region could be hydrolysed by cathespin D. Indeed the -Leu-Val- peptide bond at position 645-646 is highlighted by the PEPTIDESORT computer program as being a probable cathepsin D recognition site. This site is close to the position of three of the point mutations shown to co-segregate with certain forms of Familial Alzheimer's Disease (FAD). Cleavage within this region as well as the -Glu-Val- bond at positions 593-594 could account for the size of band 3 in Table 5. The FAD mutations at this site could augment the rates of APP cleavage within this region by cathepsin D.

Finally, a double mutation of APP from -Lys-Met- to -Asn-Leu-at positions 595-596 also cosegregates with familial AD. This mutation makes it unlikely that a specific amyloidogenic protease exists with specificity for cleavage about the -Lys-Met- bond, since the mutation would be expected to abrogate rather than augment cleavage by such an enzyme. Rather, the likelyhood is increased that the primary endoproteolysis yielding beta-amyloid occurs adjacent to and preferably N-terminal to this dipeptide. The -Glu-Val- bond represents the closest N-terminal site left unaffected in the S1 and S1' positions.

Conceivably, the -Asn-Leu- mutation at S2'and S3' sites to the -Glu-Val- scissile bond could augment cleavage by cathepsin D. To test whether this was the case, we compared the capacity of both cathepsin D and the P-2 enzyme peak B to hydrolyze the Substrate N-dansyl-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-NH2 (SEQ ID NO: 7), and a similar peptide in which the K-M pair was replace with NL thereby mimicking the above described FAD mutation (Figure 14). While both enzymes cleaved the wild type peptide at the M-D and EV bonds in longer time frames, very little cleavage was observed in the short incubation time shown in Figure 14. By contrast, cleavage of the mutant peptide by both enzymes occurred with initial velocities between 30 to 50 times faster than observed with the wild type peptide. The single metabolite thereby generated exhibited a retention time of 4.4 min, and had not been seen previously using the wild type peptide. This unidentified product must therefore result from hydrolysis either between the N-L pair or C-terminal to the L residue. In either case, this result is consistent with the notion that the -NL- mutation observed in this particular early onset FAD causes enhanced rates of beta-amyloid formation by providing a site that is more rapidly cleaved by the amyloidogenic protease cathepsin D. The increased rates of beta-amyloid accumulation that could result, could trigger the early onset form of Alzheimers Disease linked to this APP mutation.

The identification of cathepsin D as a serious candidate for the primary amyloidogenic protease of Alzheimer's Disease, significantly aids the effort of development of therapeutic inhibitors for the disease. For exam-

ple specific cathepsin D inhibitors could provide therapeutic benefit by inhibiting the toxic accumulation of beta-amyloid. The new information provided herein makes the comparatively straightforward to rationally design tight-binding inhibitors as has been accomplished for the design of novel inhibitors of other aspartic proteases such as renin and HIV-protease.

Alternatively cathepsin D can now be adapted for use in a high throughput screen using an in vitro peptidase assay so as to identify therapeutic inhibitors through random or semi-random search of chemical libraries. A suitable assay for such purposes could include the N-Dansyl-peptide assay described in Examples 2 and 3 of the present invention.

Example 10. Identification of a serine protease with specificity for C-terminal APP processing.

Table 4, showed that human brain contains serine proteases capable of C-terminal processing of recombinant APP, and that it some cases these serine proteases were inhibitable with aprotinin. To attempt a more facile isolation of such proteases, an alternate isolation scheme was devised (Example 1, Method 2) incorporating affinity purification on aprotinin-sepharose as an early step.

Application of this procedure for the further purification of the P-2 fraction was successful in the isolation of APP degrading activity (Figure 15). The active fractions recovered from the aprotinin-sepharose column by acid elution were further purified on a mono-Q column (Figure 16). Active fractions (Figure 16a) exhibited the capacity to form APP C-terminal fragments of 11 kDa, 14 kDa and 18 kDa, when analysed by immunoblot with a polyclonal antibody to the APP C-terminus (Figure 16b). The smallest products co-migrated with the recombinant C-100 standard. Reassay of APP degradation in the active fractions using an anti-beta-amyloid monoclonal antibody C286.8A led to the detection of the same three product bands (Figure 16b). Since the antibody C286.8A recognizes the first seven amino acid residues of the beta-amyloid peptide, as in Example 6(iii), this experiment shows that all three products contained full length beta-amyloid.

One or more of these product peptides could be amyloidic or give rise to beta-amyloid by further processing of these peptides C-terminal to the beta-amyloid region. The serine protease activity invloved in formation of these products could therefore play a role in amyloidosis.

The enzymic activities which formed the 11, 14 and 18 kDa product bands described above eluted as a broad peak from mono-Q and could perhaps have resulted from the action of more than one protease. Based on the recovery of A280 nm absorbing components from the mono-Q column, three different pools of proteolytic activity were prepared from the mono-Q column fractions termed pool X, Y and Z (Method 2, Example 1).

Enzymatic activity was recovered in the void volume during chromatography of each pool on superdex 75 (data not shown), consistent with an apparent Mr >75 kDa, although possible protein aggregation during chromatography cannot be ruled out. Pool Y represented the purest pool when analysed on SDS-PAGE, and exhibited a major stained band at Mr of approx 100 kDa. Pool Y was selected for further characterization. The pH dependence for APP hydrolysis by pool Y showed an optimum between pH 7 and 9 (Figure 17a), and the enzyme activity was gradually inhibited by increases in sodium chloride concentration beyond 42 mM (Figure 17b). Studies of the inhibitor sensitivity of the enzyme (Figure 18a), confirmed that it was a serine protease, being inhibited by PMSF and aprotinin but unaffected by pepstatin A, E-64 or EDTA (Figure 18a). The serine protease inhibitor benzamidine was without effect on the enzyme, suggesting that it was unlikely to be a trypsin-like endoprotease. More likely the enzyme is of the chymotryptic family with specificity for cleavage of substrates containing a neutral hydrophobic residue at the S1 subsite.

Accordingly, further inhibitor studies (Figure 18b) showed that the activity of the pool Y protease was strongly inhibited by chymotrypsin inhibitor II, alpha-2-antiplasmin and TPCK. Weak inhibition was also observed with chymostatin and alpha-1-antichymotrypsin, but TLCK, did not inhibit at all. Cathepsin G has been suggested by others to play a role in APP processing, however immunoblot analysis of the pool Y protease fraction using a polyclonal antibody to cathepsin G failed to detect the presence of this serine protease. N-terminal sequening of the 11, 14 and 18 kDa will identify the cleavage sites and is already ongoing.

Example 11. Design of therapeutic cathepsin D inhibitors.

This example utilizes the nomenclature of Schechter et al., 1967, Biochem Biophys Res Comm. 27:157, to describe peptide specificity, wherein the amino acids in the substrate which flank the scissile bond are numbered according to their position relative to the peptide bond being cleaved by the enzyme. Peptide substrate amino acid side chains N-terminal to the scissile bond are numbered consecutively as P1 to Pn with increasing distance from the scissile bond. Peptide substrate amino acid side chains C-terminal to the scissile bond are numbered consecutively as P1' to Pn'. The P1 and P1' amino acid side chains correspond to the amino acids involved in formation of the peptide bond which is to be cleaved. The side chains P1 to Pn and P1' to Pn' are

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envisioned to form specific interactions with a corresponding series of enzyme subsites S1 to Sn and S1' to Sn respectively. The interactions between the P side chains and corresponding S subsites contribute to the binding energy for stabilization of the protease-substrate complex, and thus confer specificity to the interaction.

5 The approach taken to the development of peptidomimetic inhibitors could utilize either the N-dansyl peptide substrate assays of Examples 2 and 3, or the assay of holo-APP degradation described in Example 8, to make enzymologic measurements, in conjunction with purified cathepsin D.

Quantification of optimal peptide length and sequence for proteolytic cleavage by cathepsin D in vitro. Starting with a dodecapeptide peptide of sequence Dns-Ile-Ser-Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-NH2

10 (SEQ ID NO: 7), the effect of shortening of the peptide either from the N-terminus or the C-terminus on the apparent kinetic parameters for enzymic hydrolysis would be determined at acidic pH and optimal ionic strength. The effect on Km and Vmax for hydrolysis of variation in the amino acids at each position in the peptide of optimal length would be determined.

Inhibitor synthesis. Peptidomimetic compounds would be synthesized containing essential amino acid se-

15 quences necessary for optimal cleavage (from 1 a,b above), and the appropriate spacer. The amino acid sequences in these peptides could be the same as those observed around the cleavage site in the APP substrate, eg. Glu-Ile-Ser-Glu-Val-Lys-Met-Asp (SEQ ID NO: 4) and Trp-His-Ser-Phe-Gly-Ala-Asp-Ser (SEQ ID NO: 5) or alternatively selected from those sequences found to confer optimal binding to cathepsin D based on studies of their potency for in vitro inhibition of cathepsin D. In the case of Glu-Ile-Ser-Glu-Val-Lys-Met-Asp (SEQ ID

20 NO: 4) and Trp-His-Ser-Phe-Gly-Ala-Asp-Ser (SEQ ID NO: 5) the P1-P1' bond is E-V, and F-G respectively. Peptidic inhibitors would be synthesized that contain either the above sequences or sequences exhibiting optimal cathepsin D inhibition (including shorter variants perhaps containing N- and/or C- substitutions), in which the -CO-NH- atoms of the peptide bond between P1 and P1' are replaced with any of the following standard spacer groups and using appropriate synthetic routes so as to obtain any possible stereo-chemical configur-

25 ation thereof: reduced amide, hydroxy isostere, ketone isostere, dihydroxy isostere, statine analogs, phosphonates or phosphoamides, reversed amides. Most of these inhibitors would function as transition state analogs. The potency of these first generation compounds as determined using either of the in vitro assays of the present invention (N-Dansyl-peptide assay of holo-APP degradation assay) could be optimized by any or all of the following:

30   i) Addition or deletion of flanking amino acid residues;

    ii) Alteration of the type of amino acid side chain (D or L) at each position in the inhibitor;

    iii) N- and C-terminal substitution with blocking groups such as boc or acetyl (N-terminally), or O-Me , O-benzyl, N-benzyl (C-terminally).

Beside the inhibitors rationally developed according to the above methods, other known cathepsin D inhibitors

35 either in whole or in part could be used as therapeutic inhibitors for Alzheimers Disease, or as starting points for optimization of inhibitory potency and the development of new derivatives for therapy of Alzheimer's Disease. Such inhibitors include: 1-Deoxynojirimicin (Lemansky et al., 1984, J. Biol Chem. 259:10129); Diazoacetyl-norleucine methyl ester (Keilova at al., 1970, Febs Lett, 9:348); Gly-Glu-Gly-Phe-Leu-Gly-Asp-Phe-Leu (SEQ ID NO: 6) (Gubenseck et al., 1976, Febs Lett, 71:42); Pepsin inhibitor from Ascaris (Keilova et al., 1972,

40 Biochem Biophys Acta. 284:461); pepstatin (Yamamoto et al., 1978, European Journal of Biochemistry 92:499).

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SEQUENCE LISTING

(1)   GENERAL INFORMATION:

   (i)   APPLICANT: Paul P. Tamburini and Robert N. Dreyer

   (ii) TITLE OF INVENTION: Methods for Detecting
                                 Beta Amyloid Precursor Protein
                                 Processing Enzyme

   (iii)   NUMBER OF SEQUENCES: 8

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   (v)   COMPUTER READABLE FORM:

         (A)   MEDIUM TYPE: Floppy diskette
         (B)   COMPUTER: IBM PC
         (C)   OPERATING SYSTEM: MS-DOS
         (D)   SOFTWARE: Word Perfect 5.1

   (vi)   CURRENT APPLICATION DATA:

         (A)   APPLICATION NUMBER:
         (B)   FILING DATE:
         (C)   CLASSIFICATION:

   (vii)   ATTORNEY/AGENT INFORMATION:

         (A)   NAME: Jae H. Kim, Esq.
         (B)   REGISTRATION NUMBER: 32,907
         (C)   REFERENCE/DOCKET NUMBER: MTI 224

(viii)   TELECOMMUNICATION INFORMATION:

    (A)   TELEPHONE: (203) 937-2340
    (B)   TELEFAX: (203) 937-2795

(2) INFORMATION FOR SEQ ID NO: 1

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 16 amino acids
       (B) TYPE: amino acid
       (C) TOPOLOGY: linear

    (ii) SEQUENCE DESCRIPTION: SEQ ID NO:1

      Ile Ser Glu Val Lys Met Asp Ala Glu Phe
       1   5   10

      Arg His Asp Asp Asp Asp
                   15

(3) INFORMATION FOR SEQ ID NO: 2

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 12 amino acids
       (B) TYPE: amino acid
       (C) TOPOLOGY: linear

    (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 2

      Ile Ser Glu Val Lys Met Asp Ala Glu Phe
       1   5   10

      Arg His

36

(4) INFORMATION FOR SEQ ID NO: 3

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (C) TOPOLOGY: linear

    (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 3

        Ile Ser Glu Val Asn Leu Asp Ala Glu Phe Arg
         1   5   10

(5) INFORMATION FOR SEQ ID NO: 4

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (C) TOPOLOGY: linear

    (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 4

        Glu Ile Ser Glu Val Lys Met Asp
         1   5

(6) INFORMATION FOR SEQ ID NO: 5

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (C) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO: 5


```
Trp His Ser Phe Gly Ala Asp Ser
 1               5
```


(7) INFORMATION FOR SEQ ID NO: 6

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 9 amino acids
        (B) TYPE: amino acid
        (C) TOPOLOGY: linear

    (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 6


```
Gly Glu Gly Phe Leu Gly Asp Phe Leu
 1               5
```


(8) INFORMATION FOR SEQ ID NO: 7

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (C) TOPOLOGY: linear

    (ii) SEQUENCE DESCRIPTION: SEQ ID NO: 7


```
Ile Ser Glu Val Lys Met Asp Ala Glu Phe
 1               5                  10

Arg
```

(9)   INFORMATION FOR SEQUENCE ID NO: 8

    (i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 39 nucleotides
        (B)   TYPE: nucleic acids
        (C)   STRANDEDNESS: single strand
        (D)   TOPOLOGY: linear

    (ii)   MOLECULAR TYPE:

        cDNA to mRNA

    (iii)   PUBLICATION INFORMATION:

        (A)   AUTHORS: Kang et al.
        (B)   JOURNAL: Nature
        (C)   VOLUME: 325
        (D)   PAGE: 733
        (E)   DATE: 1987

    (iv)   SEQUENCE DESCRIPTION: SEQ ID NO: 8

        CTCTAGAACT AGTGGGTCGA CACGATGCTG CCCGGTTTG   39

Claims

1.   A method for regulating formation of beta-amyloid protein with an inhibitor of at least one protease specific

for the Precursor to the Alzheimer's Disease beta-amyloid protein.

2. The method of claim 1, wherein said inhibitor is selected from the group of inhibitors consisting of those specific for aspartic proteases and serine proteases.

3. The method of claim 2, wherein said inhibitor of aspartic proteases specifically inhibits cathepsin D.

4. The method of claim 2, wherein said inhibitor of serine proteases specifically inhibits a serine protease which is inhibited by alpha-2-antiplasmin, chymotrypsin inhibitor II, or TPCK, and which forms 11, 14 and 18 kDa APP C-terminal fragments at pH 7-9.

5. The method of claim 3, wherein said inhibitor is selected from the group consisting of 1-Deoxynojirimicin, Diazoacetyl-norleucine methyl ester, Gly-Glu-Gly-Phe-Leu-Gly-Asp-Phe-Leu (SEQ ID NO: 6), Ascaris Pepsin Inhibitor, and Pepstatin.

6. A method for preventing the formation of amyloid plaques in Alzheimer's Disease, comprising administering a therapeutic amount of an inhibitor to cathepsin D.

7. The method of claim 6, wherein said inhibitor is a transition state analog containing a reactive spacer selected from the group consisting of reduced amides, hydroxy isosteres, ketone isosteres, dihydroxy isosteres, statine analogs, phosphonates, phosphoamides, and reversed amides.

8. A method for identifying inhibitors of cathepsin D, comprising:
   a) incubating cathepsin D with a peptide substrate capable of being cleaved by cathepsin D to form a first incubate conducted under conditions at which the cathespsin D is catalytically active;
   b) incubating cathepsin D with a peptide substrate capable of being cleaved by cathepsin D in the presence of a potential inhibitor to form a second incubate;
   c) analyzing the amount of peptide products formed over a time period to calculate the product formation rate in said first and said second incubates; and
   c) calculating the reduced enzyme activity observed in the presence of the potential inhibitor, said reduction indicating inhibitory activity.

9. The method of claim 8, wherein said cathespsin D is human cathepsin D.

10. The method of claim 8, wherein said peptide substrate is N-dansylated.

11. A method for measuring the proteolytic activity of molecules capable of degrading amyloid precursor protein, comprising:
   a) incubating a physiological sample obtained from a human, in the presence of amyloid precursor protein substrate under conditions in which amyloid precursor protein degrading proteases in said sample are catalytically active;
   b) forming a gel with a portion of terminated incubation mixture of step (a);
   c) electrophoresing the gel of step (b) to obtain an electrophoretic migratory pattern representing separate polypeptide constituents;
   d) blotting said constituents of step (c) onto a membrane;
   e) contacting said blotted membrane from step (d) with anti-amyloid precursor protein antibody;
   f) reacting said blotted membrane with a second antibody that recognizes said anti-amyloid precursor protein antibody, said second antibody being coupled to a detectable ligand; and
   g) examining the intensity of staining of the blots in regions corresponding to fragments of a size sufficient to contain the beta-amyloid peptide sequence.

12. The method of claim 11 wherein said physiological sample is selected from the group consisting of brain tissue and cerebro-spinal fluid.

13. The method of claim 12 wherein said physiological sample contains cathepsin D.

14. The method of claim 11 further comprising treating said physiological sample to obtain a crude homogenate, a soluble fraction, or a detergent solubilized membrane fraction.

15. The method of claim 11 wherein said amyloid precursor protein substrate is translated from gene sequenc-

es containing point mutations.

16. The method of claim 11 wherein said amyloid precursor protein substrate corresponds to a C-terminal portion of the amyloid precursor protein.

17. The method of claim 11 wherein said anti-amyloid precursor protein antibody recognizes peptides selected from the group consisting of beta-amyloid peptides and C-terminal fragments of amyloid precursor protein.

18. The method of claim 17 wherein said C-terminal fragments comprise C-100 or beta-amyloid peptide fragments as detected by co-migration with recombinant C-100 or beta-amyloid size markers.

19. A method for identifying inhibitors of proteases specific for amyloid precursor protein, comprising:
a) forming a first incubate with a portion of a physiological sample obtained from a human and a selected amyloid precursor protein substrate, and
forming a second incubate with a second portion of said physiological sample obtained from a human, said selected amyloid precursor protein substrate and a test inhibitor;
b) terminating the incubations in step (a) after a predetermined duration;
c) forming gels with portions of the terminated reaction mixtures of step (b);
d) electrophoresing the gels of step (c) to obtain electrophoretic migratory patterns representing separate polypeptide constituents;
e) blotting said constituents of step (d) onto membranes;
f) contacting said membranes from step (e) with an anti-amyloid precursor protein antibody;
g) reacting said blotted membrane with a second antibody that recognizes said anti-amyloid precursor protein antibody, said second antibody being coupled to a detectable marker;
h) examining the intensity of staining of the blots in regions corresponding to fragments of a size sufficient to contain the beta-amyloid peptide sequence; and
i) comparing the intensities of the bands of the same size observed from said first and said second incubates.

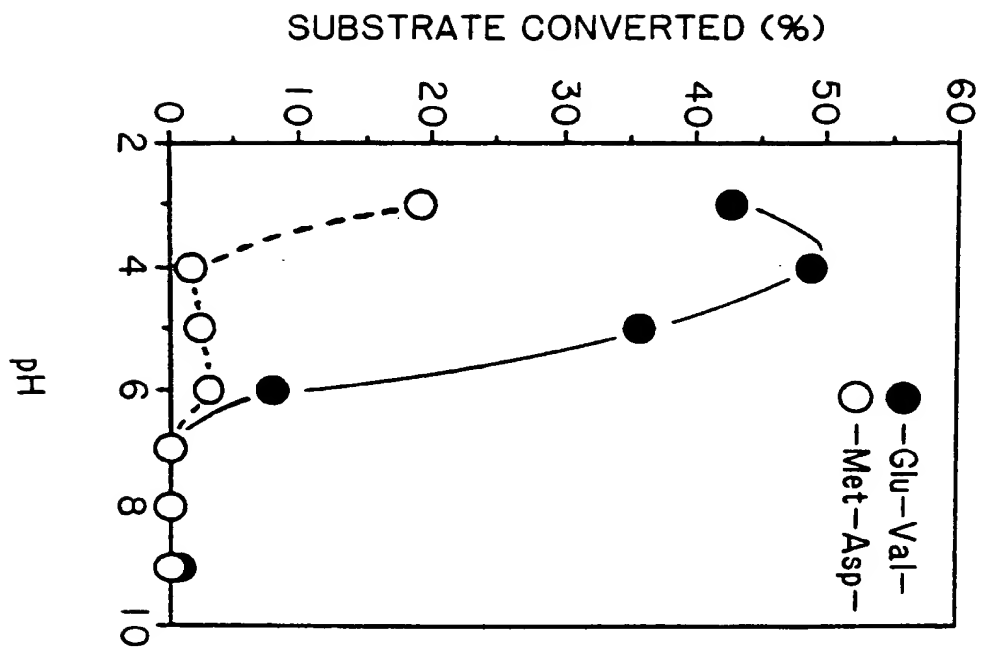20. The method of claim 19 wherein said proteases specific for amyloid precursor protein is cathepsin D.
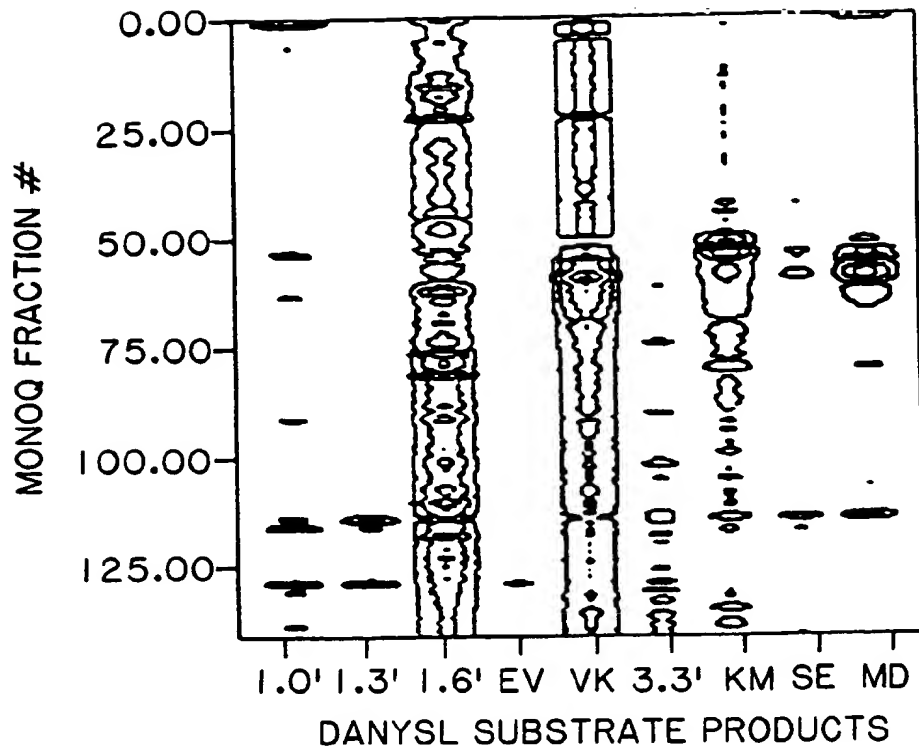
FIG. 1a
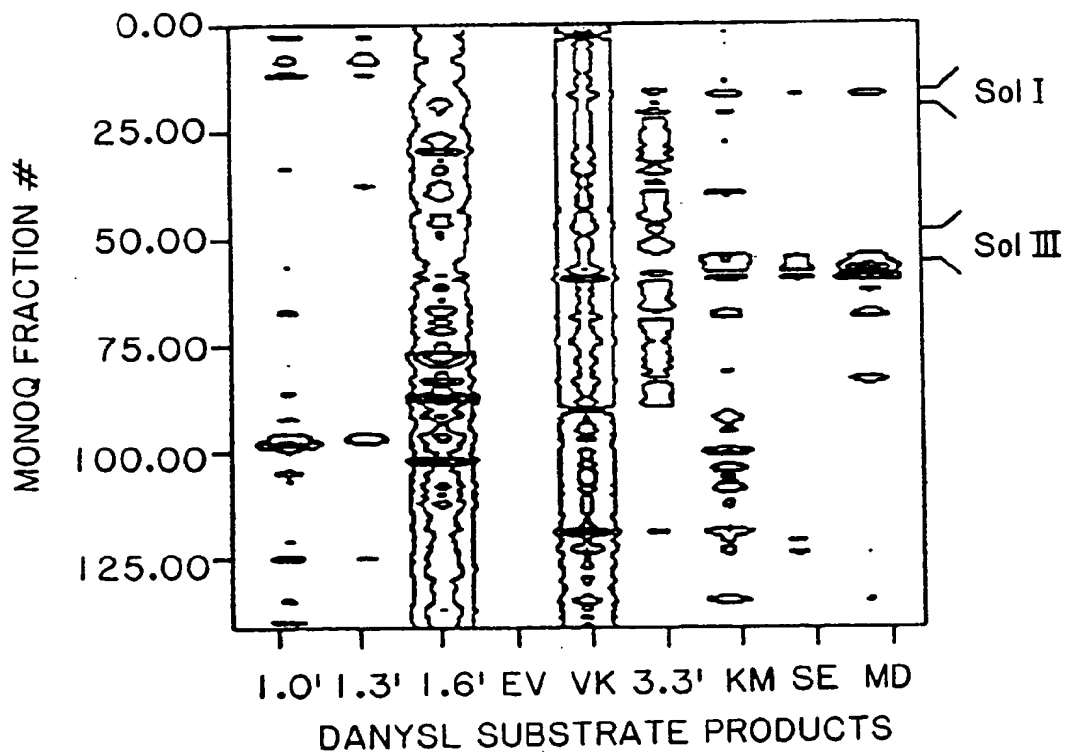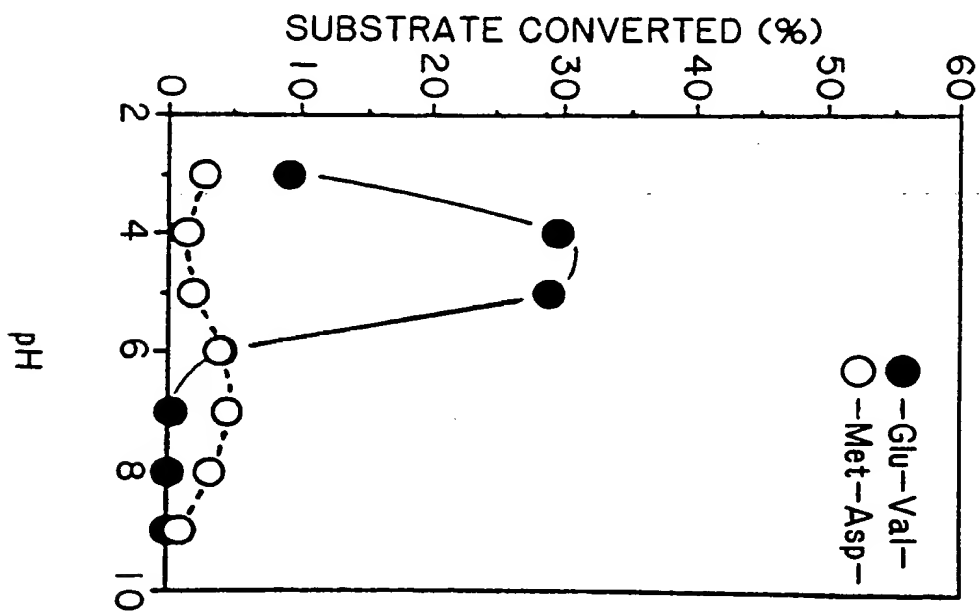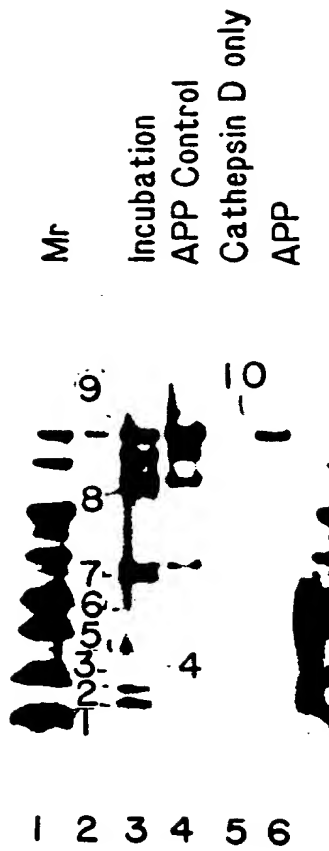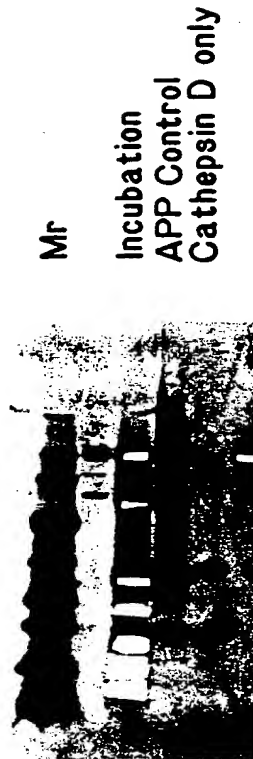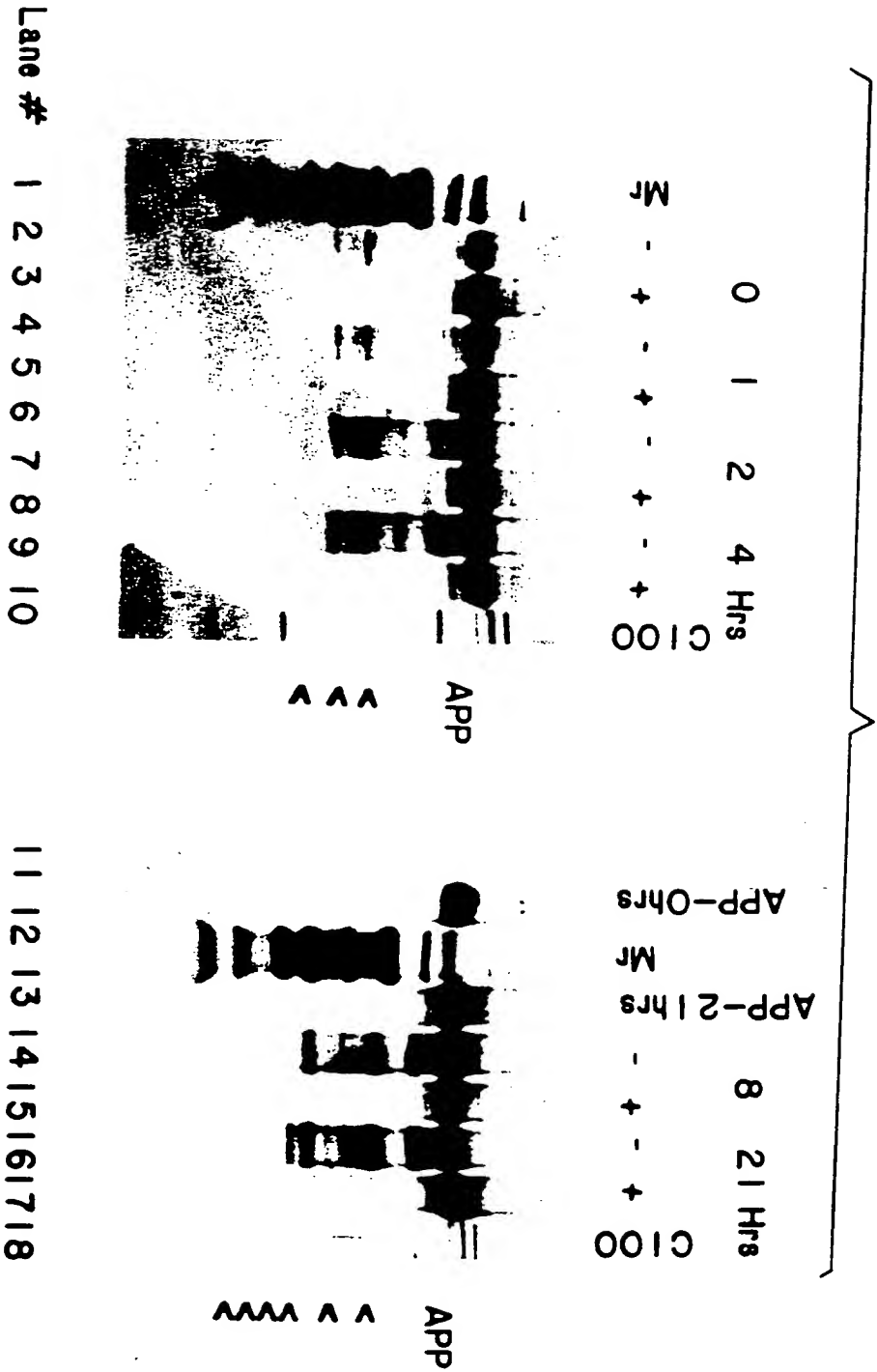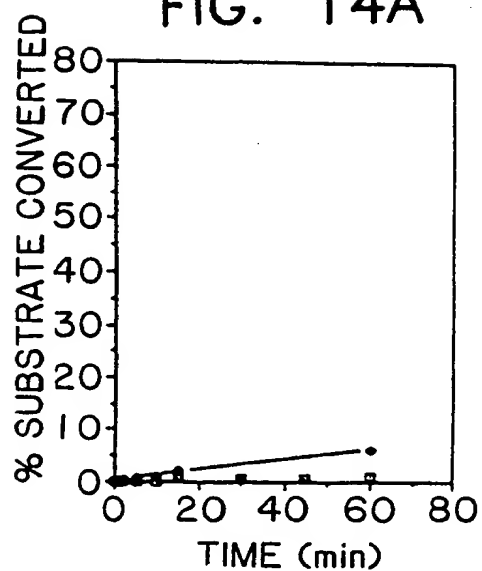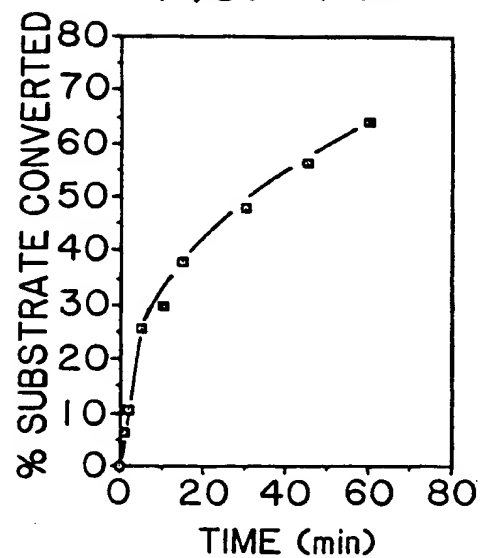

FIG. 1b
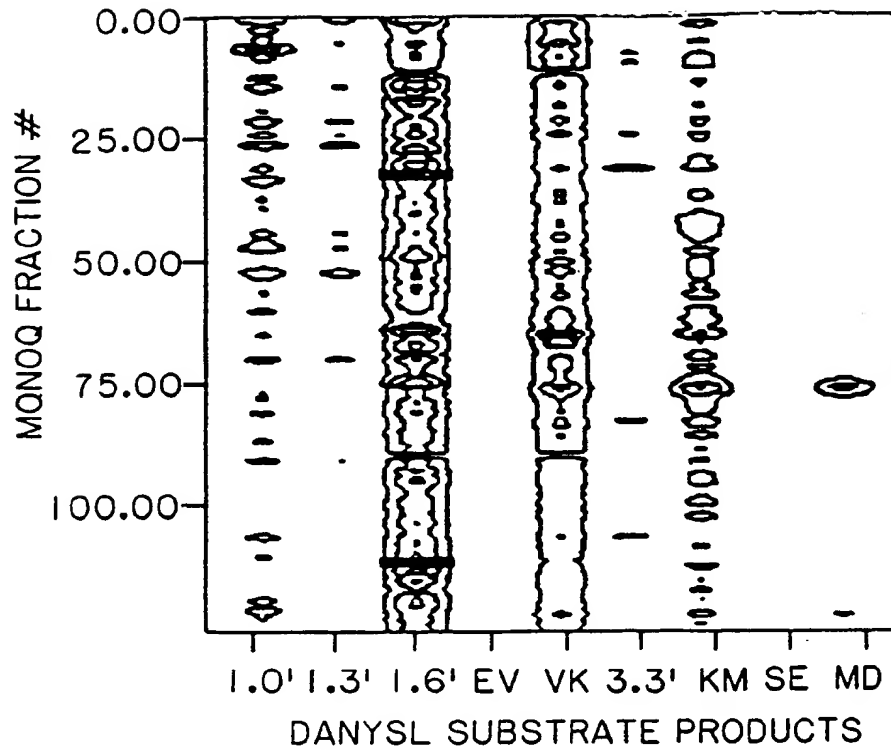
FIG. 1c

FIG. 1d

FIG. 1e

FIG. 1f

1 2 3 4 5 6

FIG. 2a

1 2 3 4 5 6 7 8

FIG. 2b

45

FIG. 2c

FIG. 2d

5 4 3 2 1

←C−100
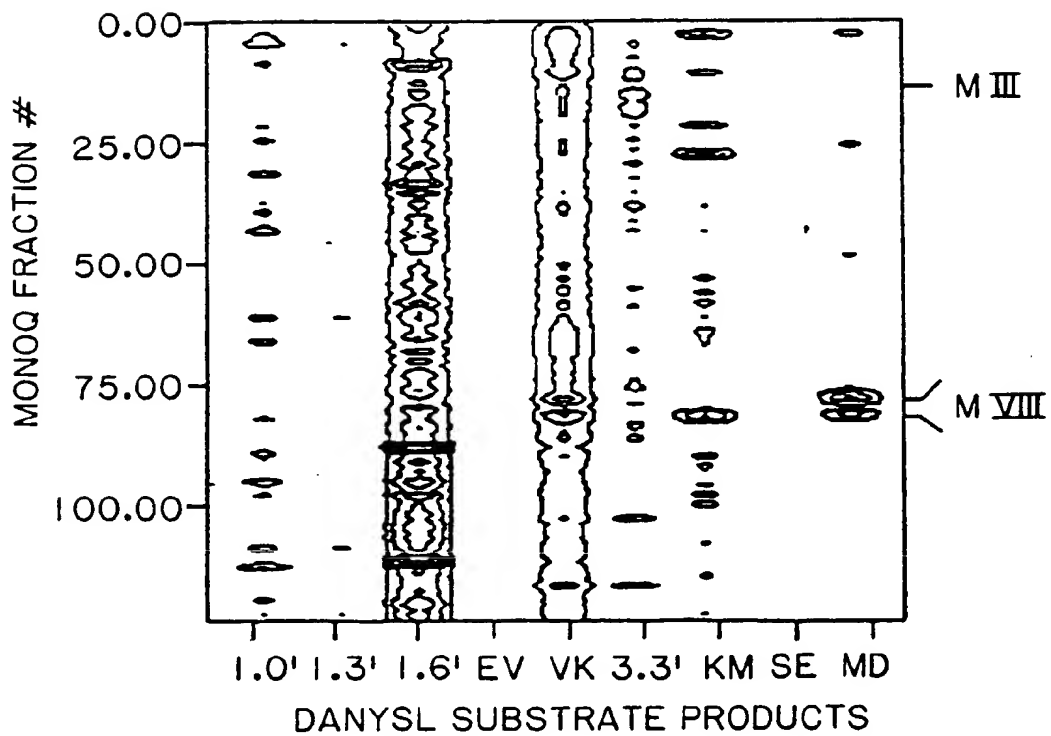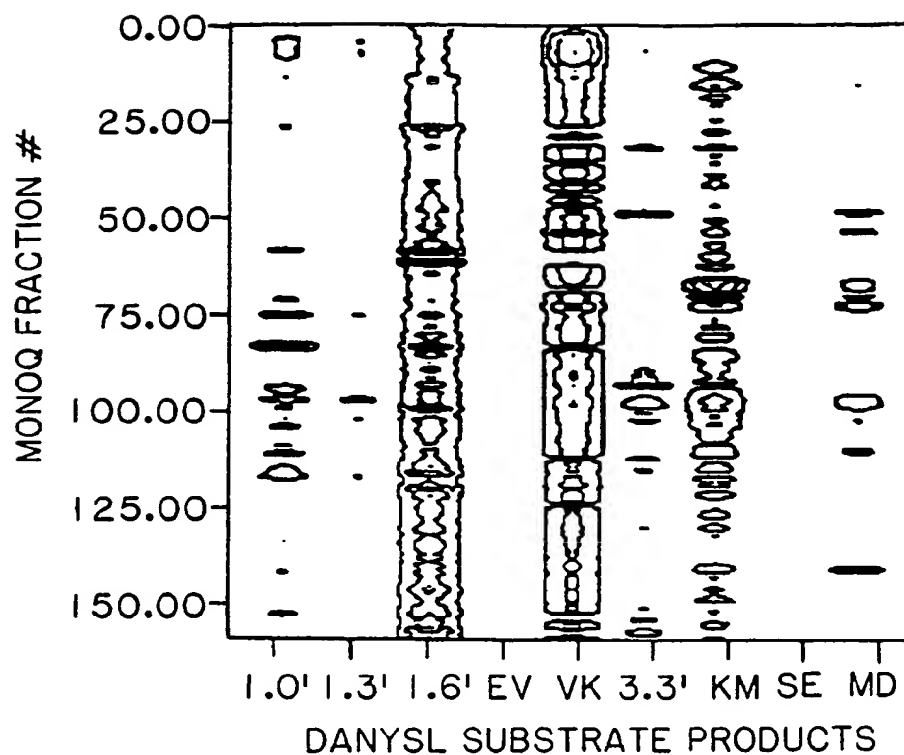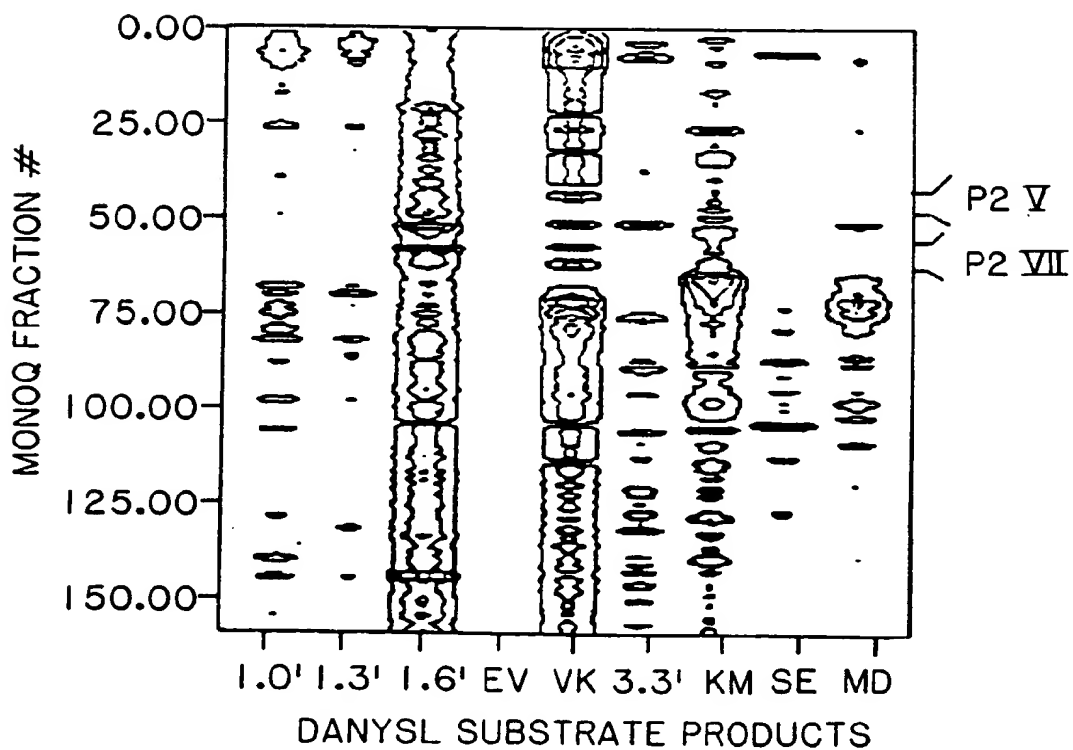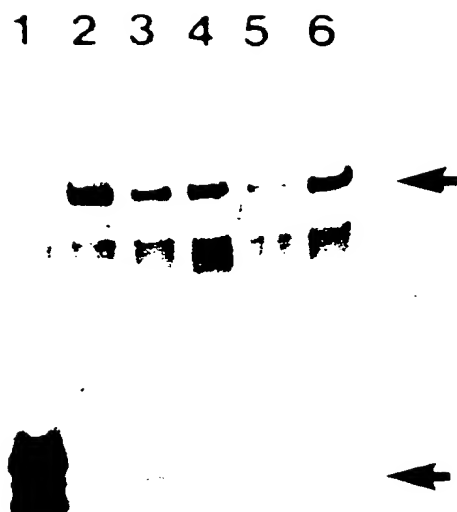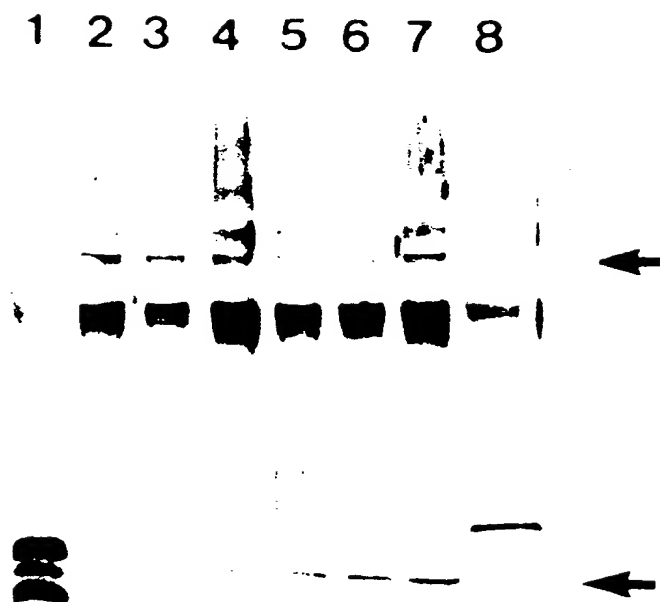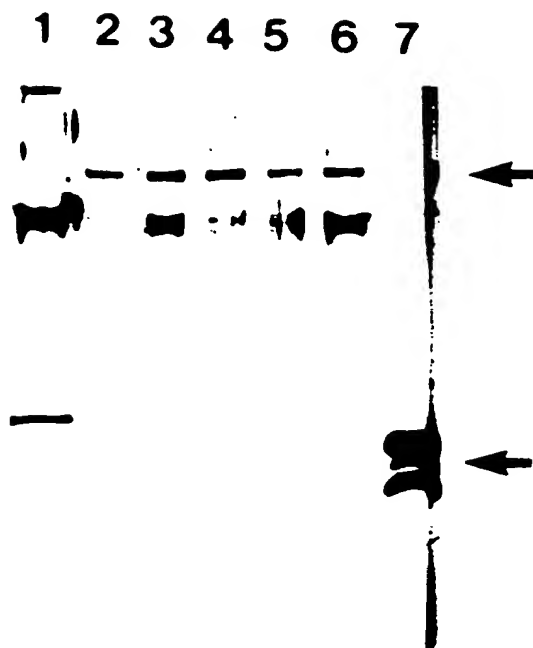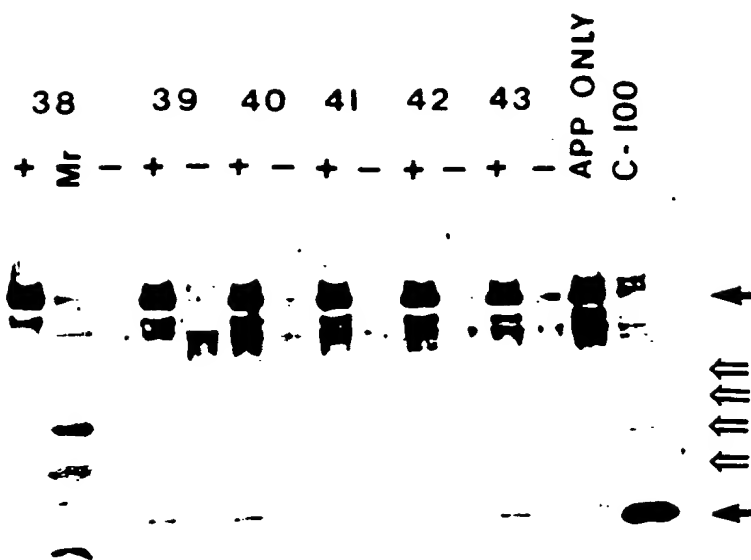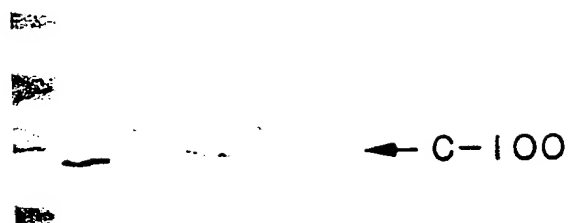
FIG. 2e

8   7   6   5   4   3   2   1

←

←

FIG. 2f
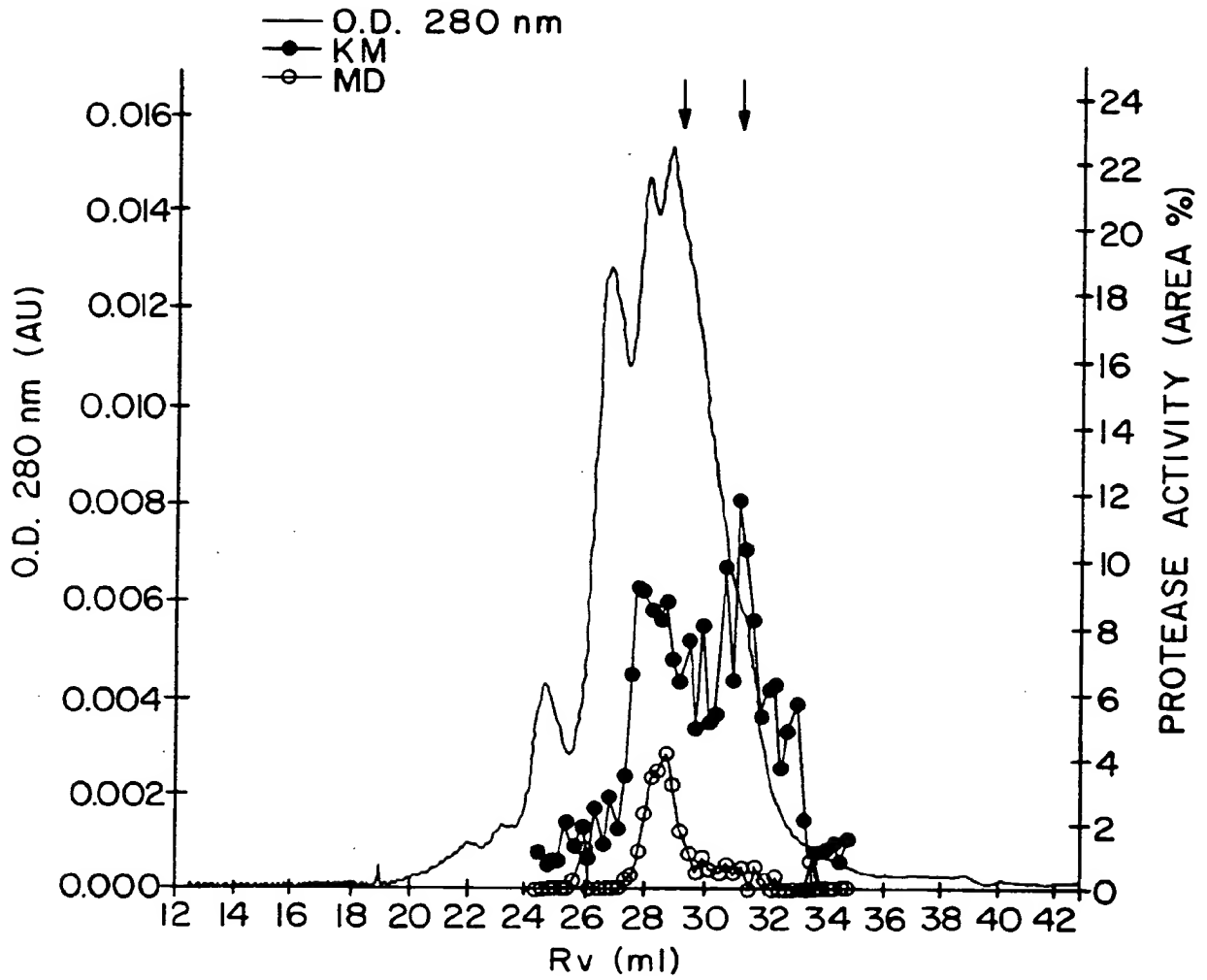
FIG. 3a
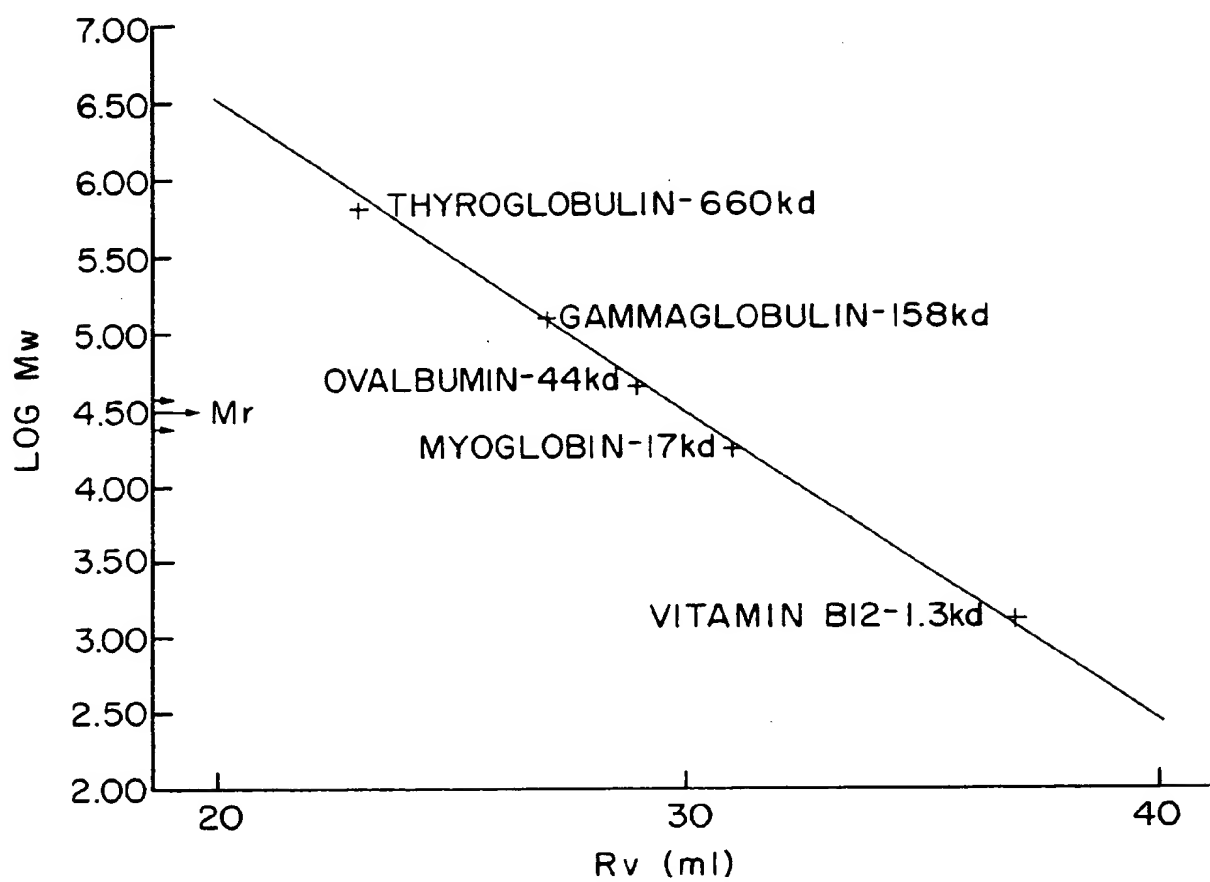
FIG. 3b

FIG. 4
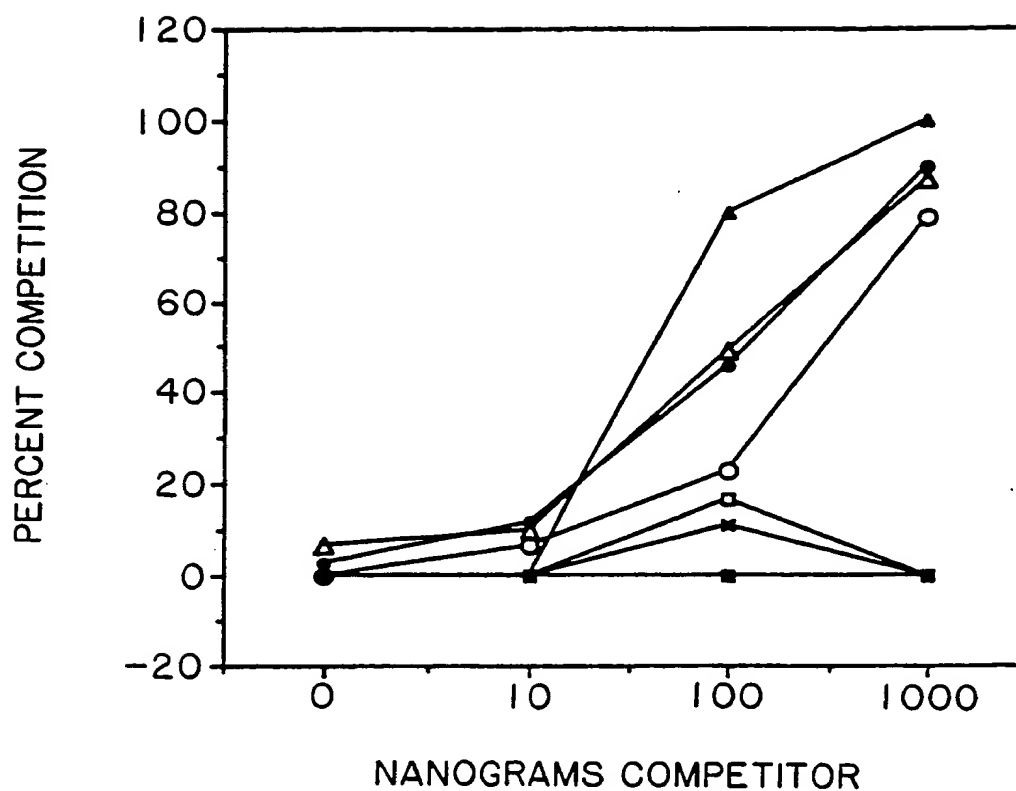
BETA—AMYLOID

FRAGMENT #

●———	1-28
▲———	1-42
○———	1-16
△———	1-7
□———	25-35
✕———	12-28

APP FRAGMENT #

■———	645-695

FIG. 5A
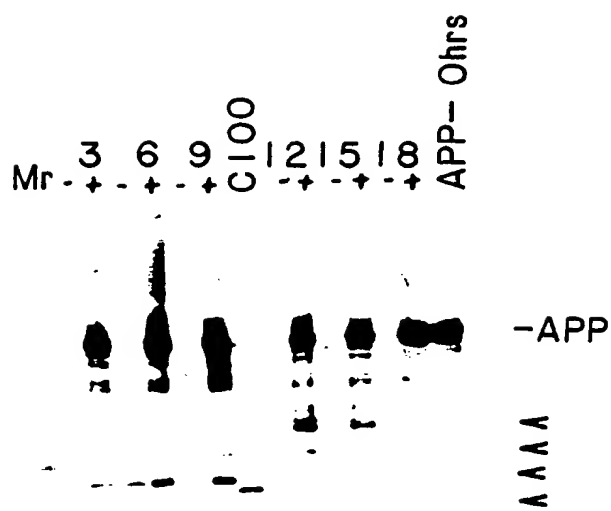
PEAK A

FIG. 5B
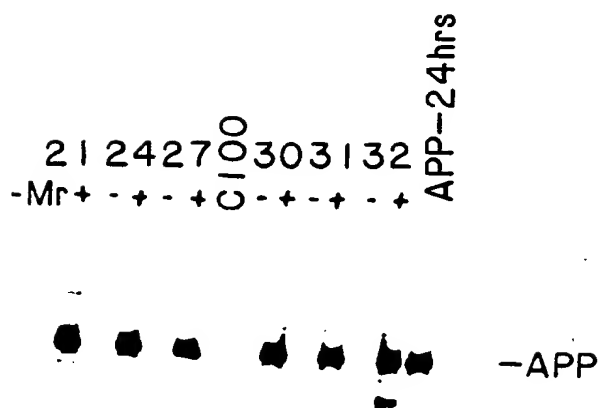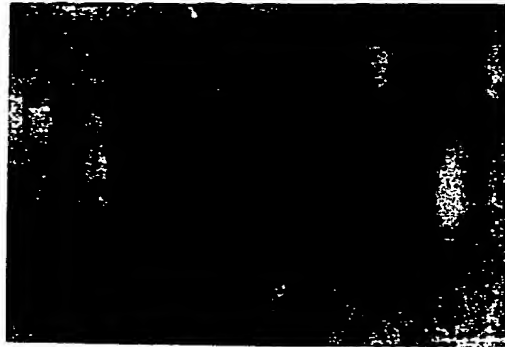
51

FIG. 5C

PEAK B

FIG. 5D

FIG. 6A

FIG. 6B

FIG. 6C

FIG. 6D

FIG. 6E

FIG. 7

FIG. 8

cathepsin D   P-2 enzyme

pH   8 7 6 5 4 3 0 0 1 0 0 9 8 7 6 5 4 3 Mr   pH

FIG. 9A

APP

cathepsin D   P-2 enzyme

NaCl (M)   0 0 1 0 0   NaCl (M)

pH 9 Mr 1.0 0.6 0.3 0.14   1.0 0.6 0.3 0.14
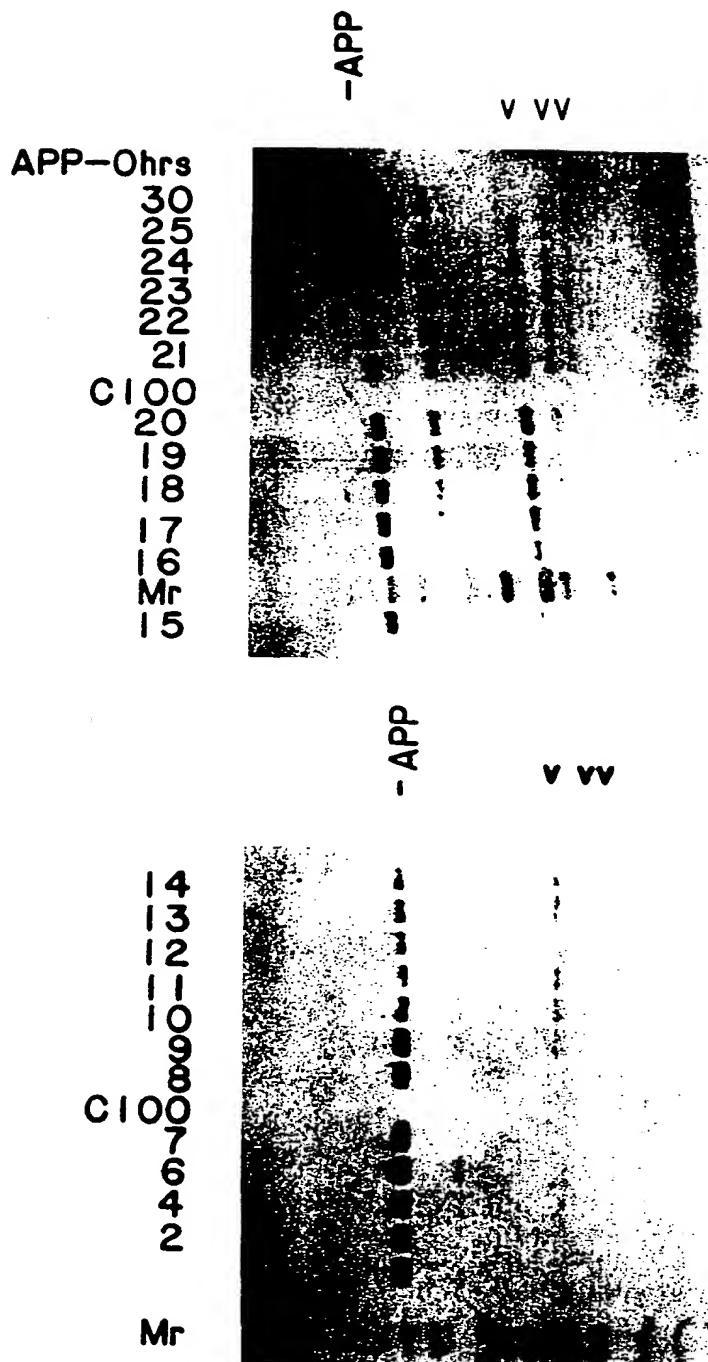
FIG. 9B

- APP

FIG. 10A

FIG. 10B

59

FIG. 1OC

FIG. 1OD

60
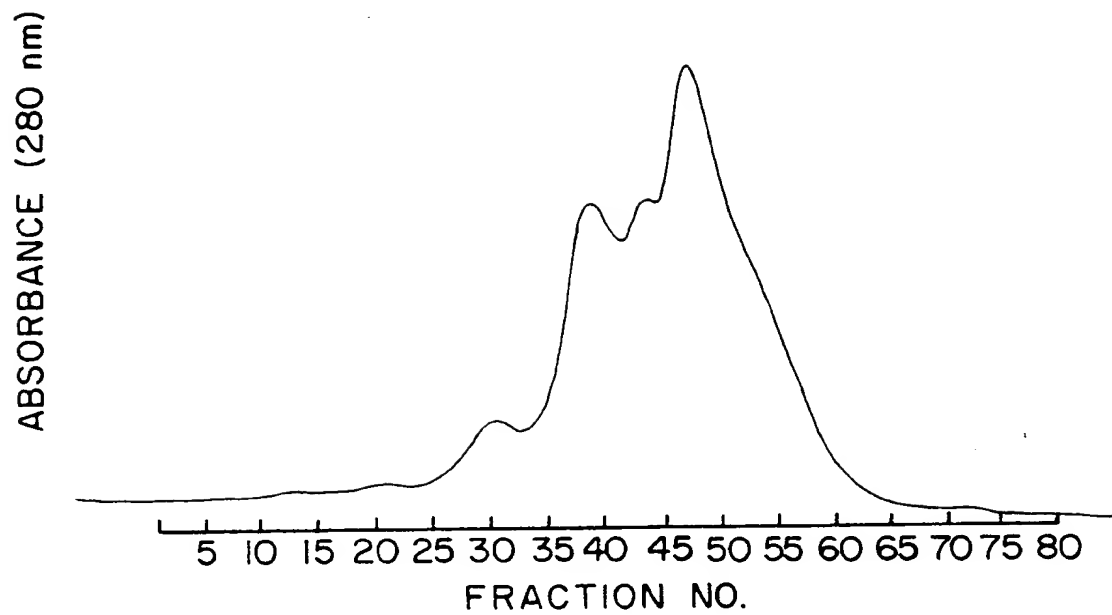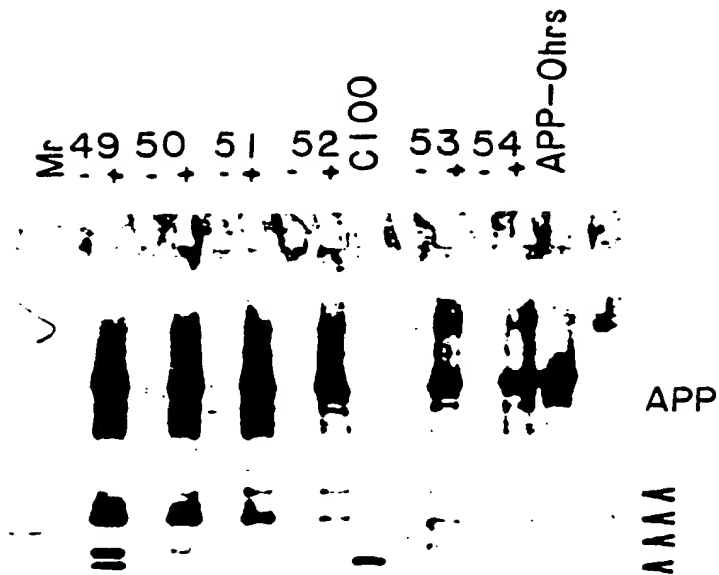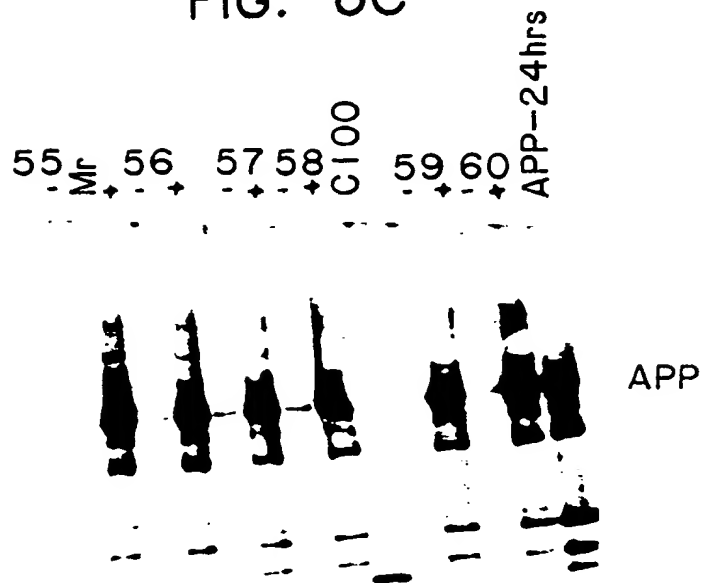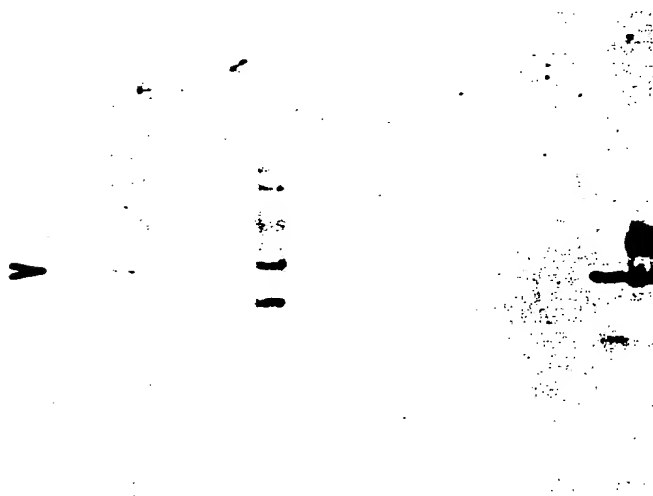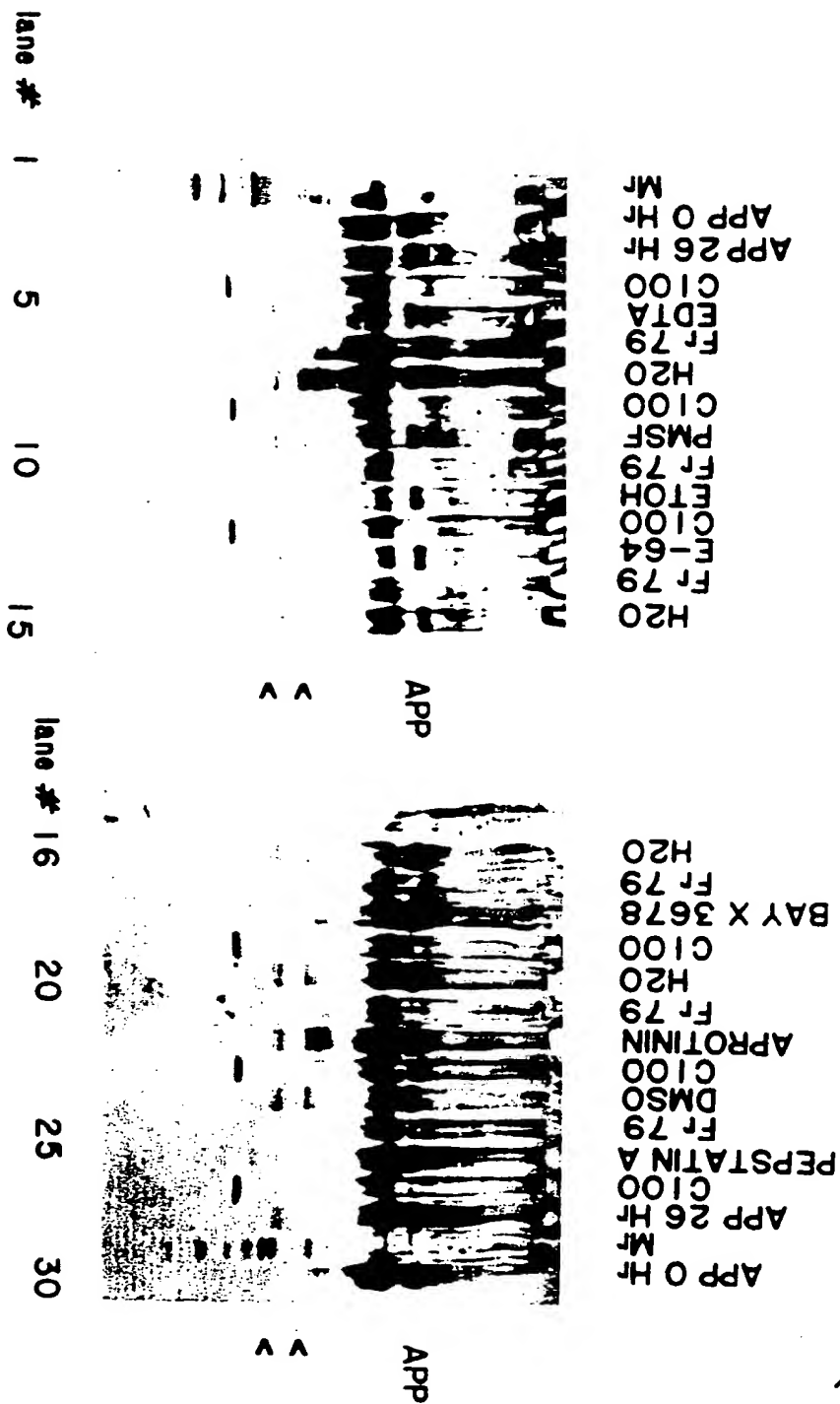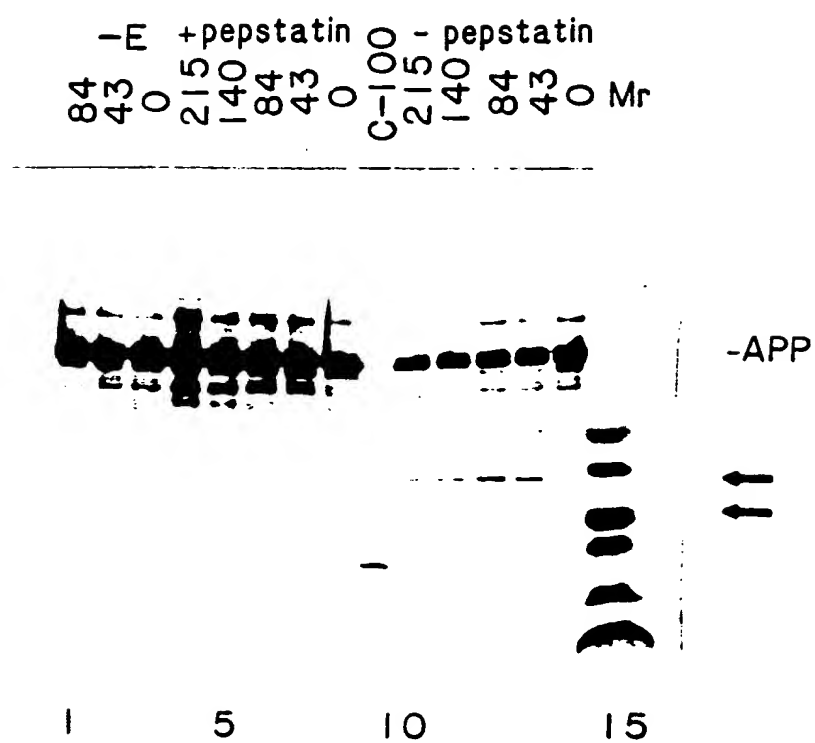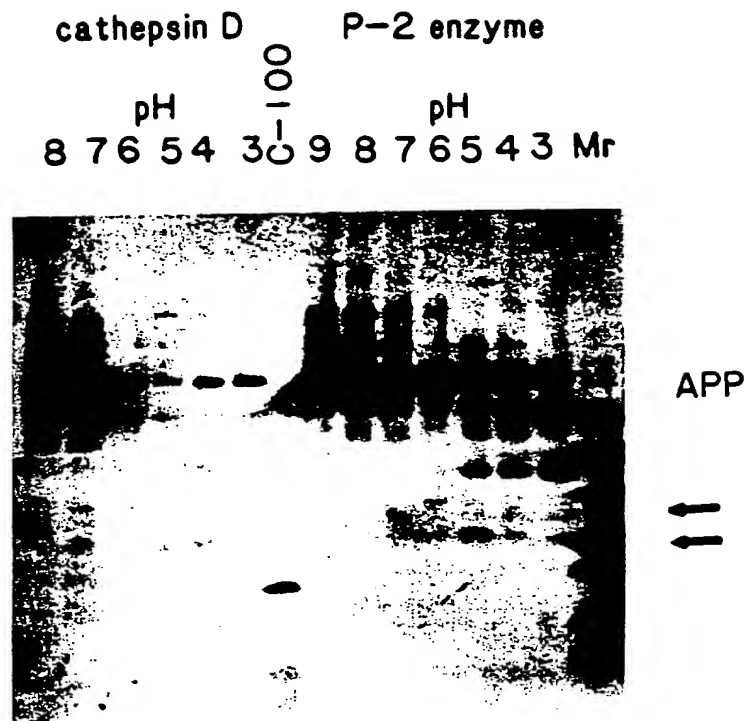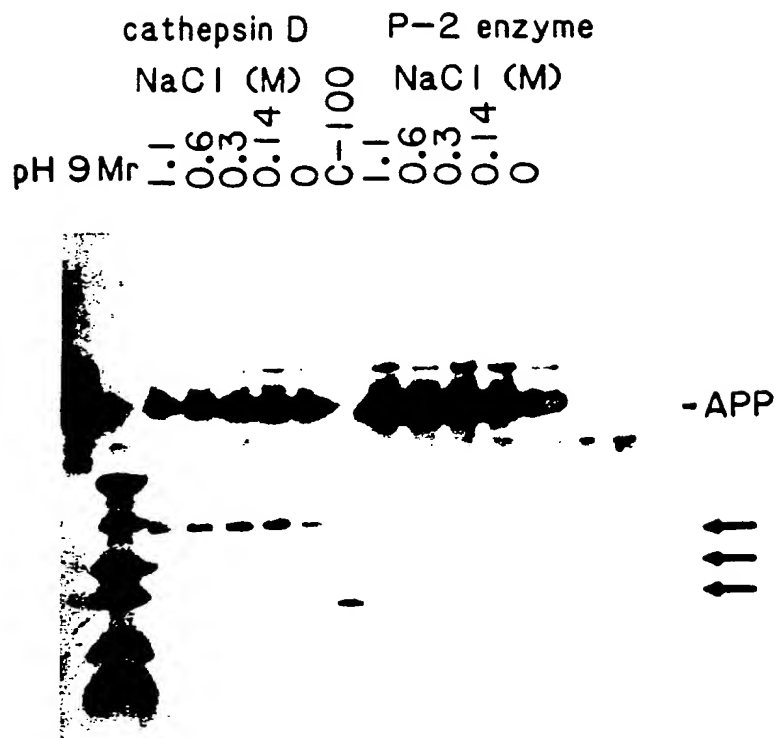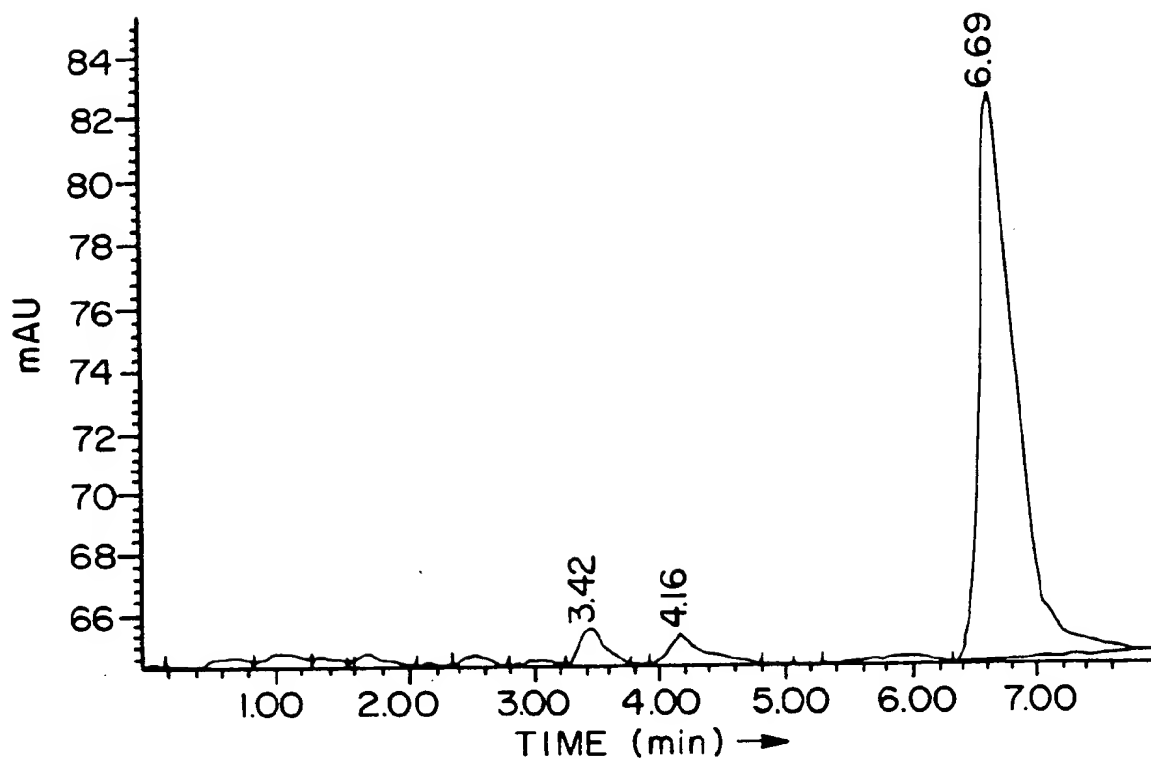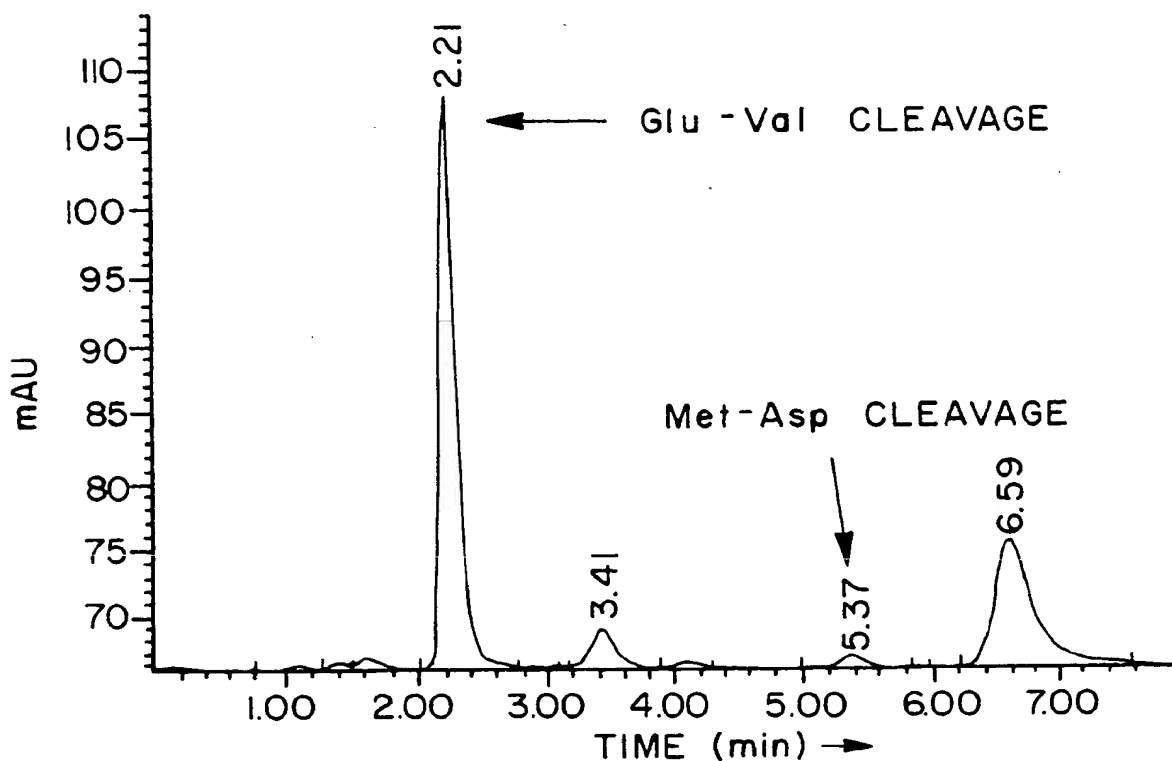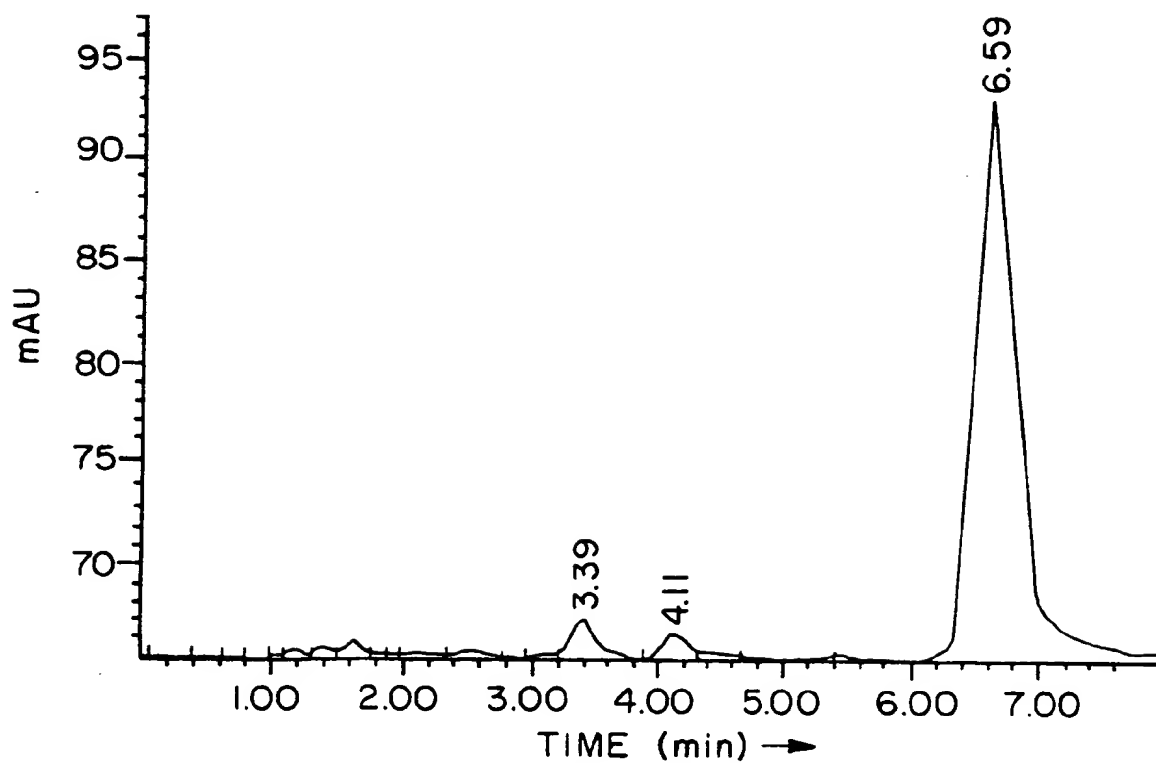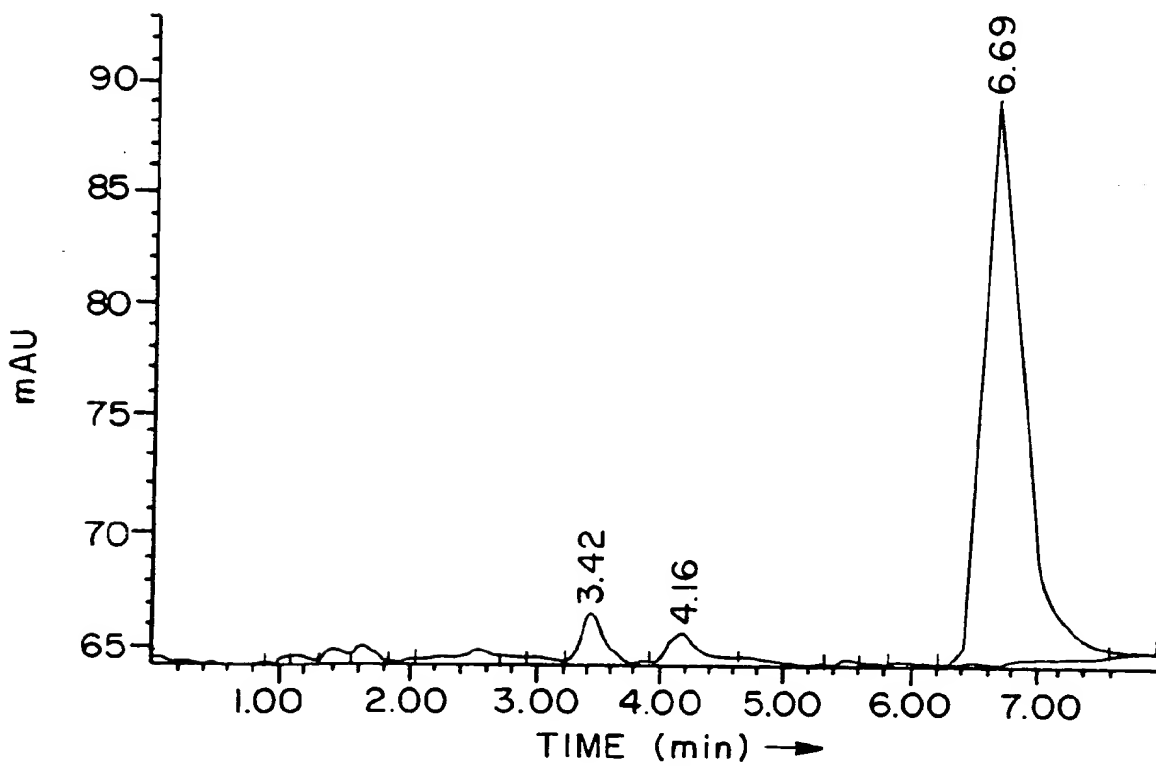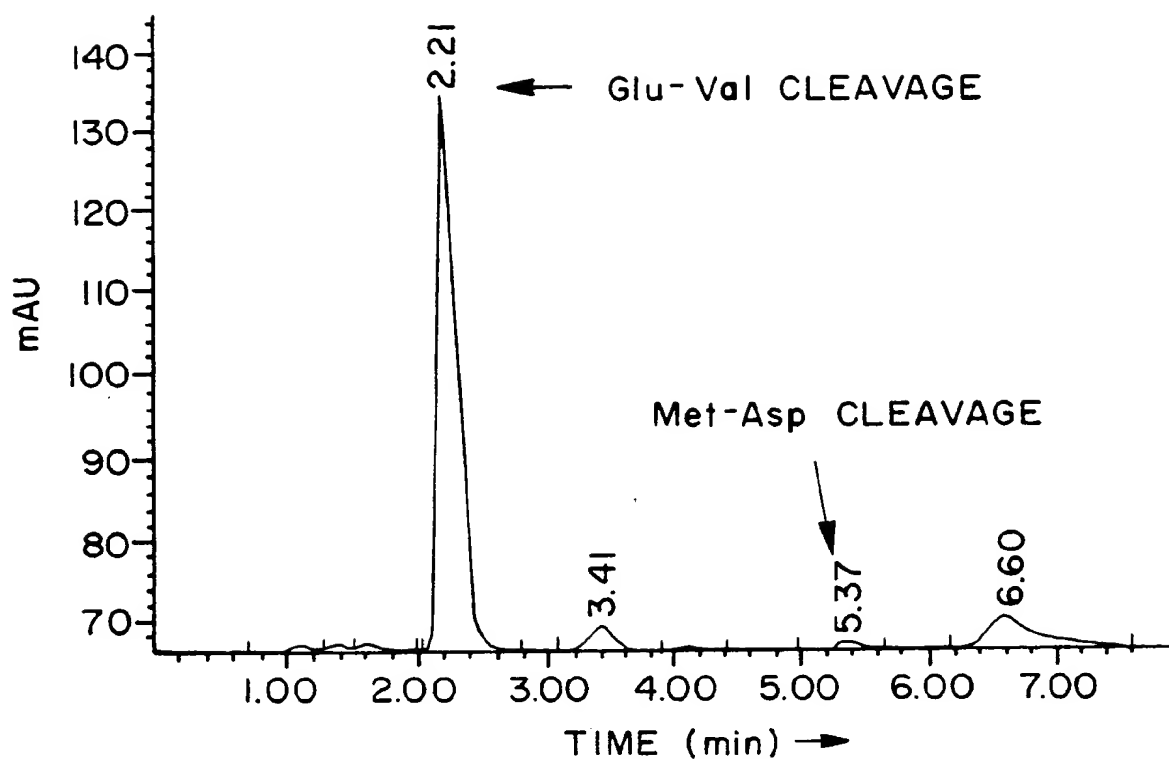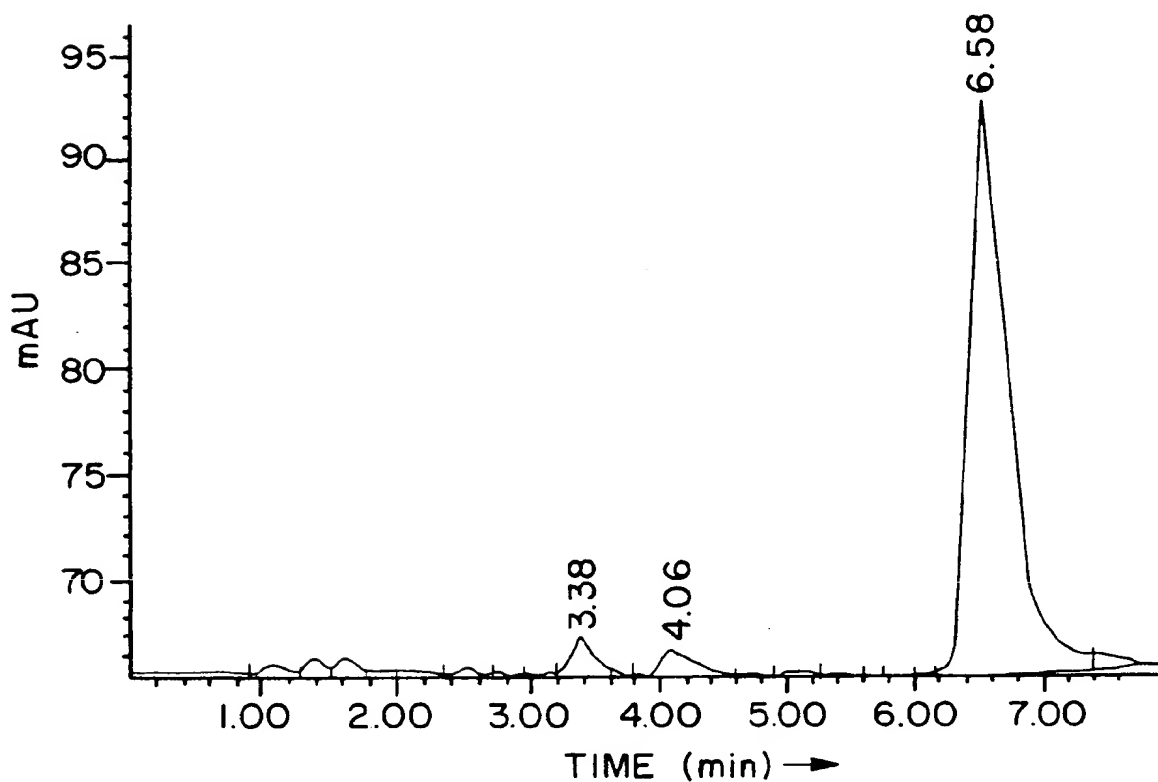
FIG. 10E

FIG. 10F

FIG. 11A

FIG. 11B
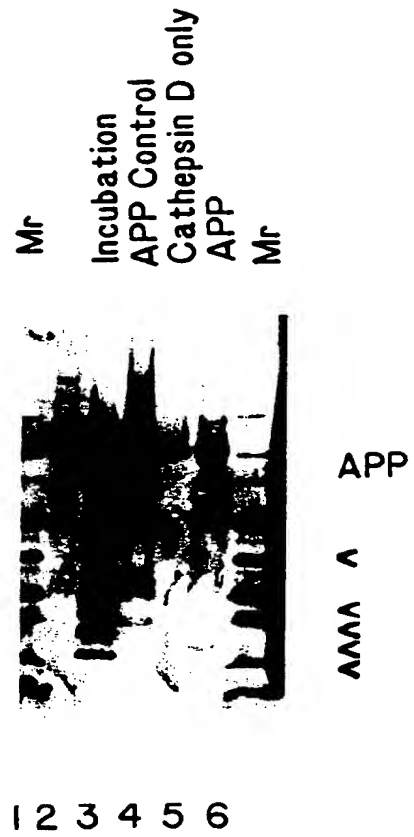
FIG. 12a   FIG. 12b   FIG. 12c

FIG. 13

FIG. 14A

FIG. 14B

FIG. 14C

FIG. 14D

65
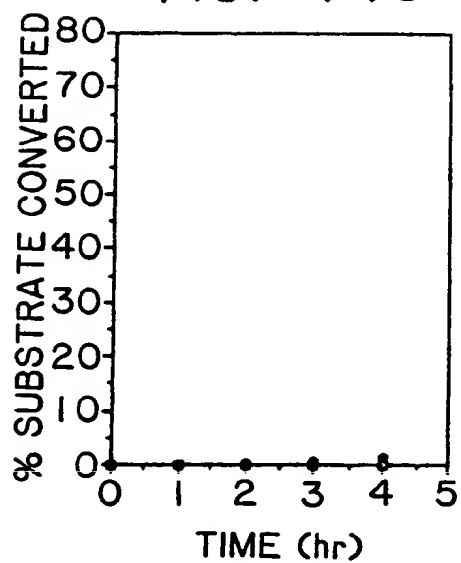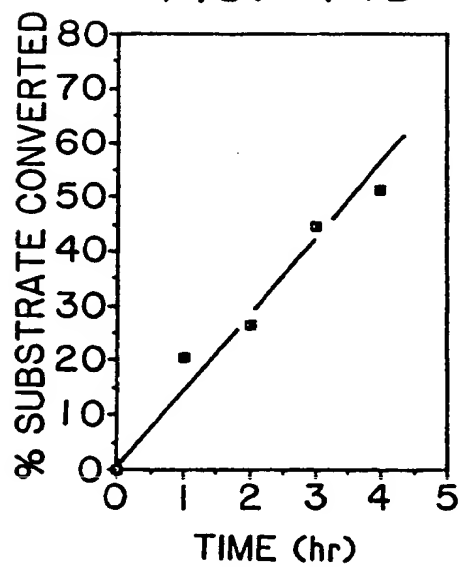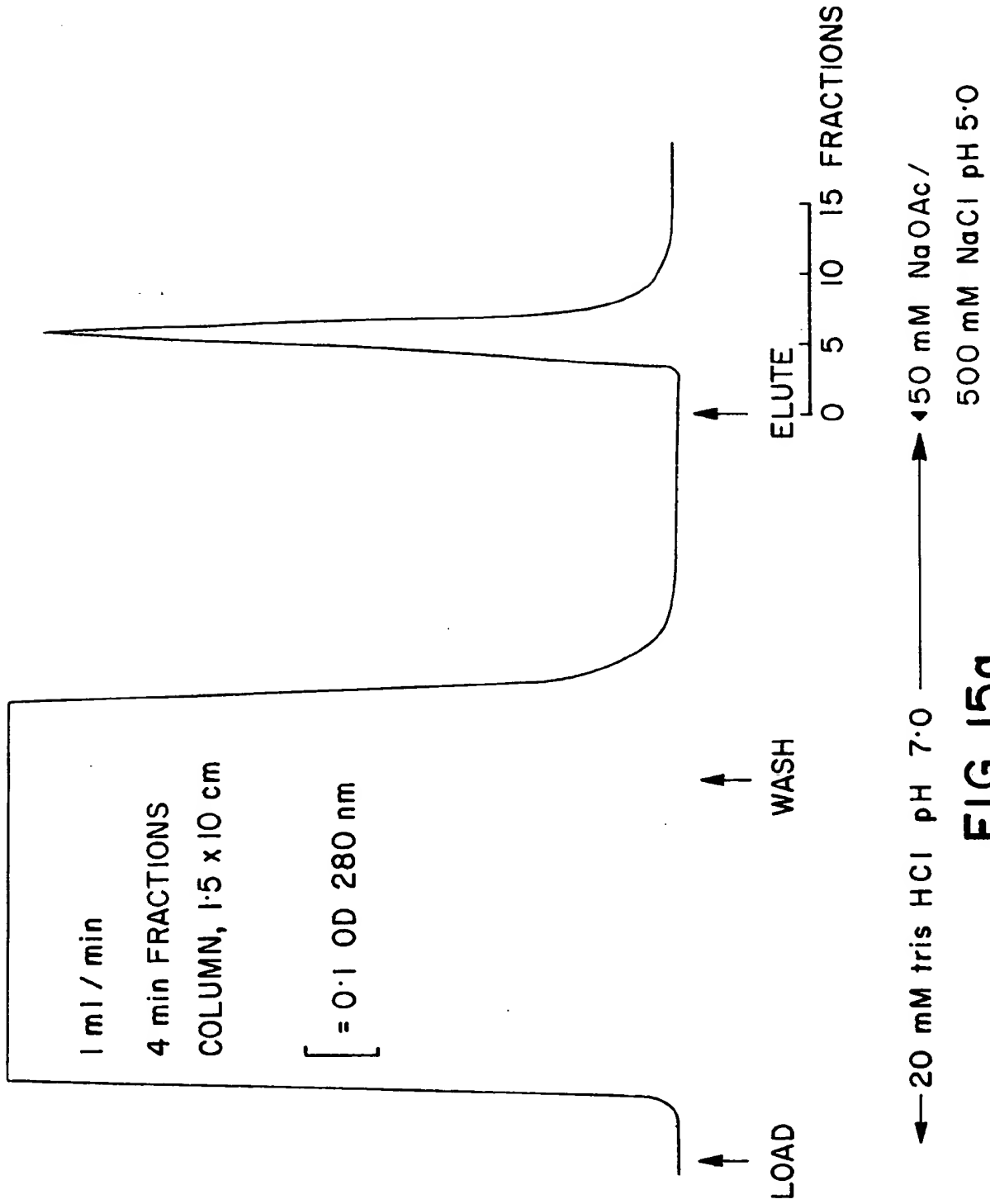
FIG. 15a

FIG. 15B-1

FIG. 15B-2

FIG. 16a

FIG. 16B

FIG. 16C

FIG. 17A

lane # 1 2 3 4 5 6 7 8 9 10 11 12

FIG. 17B

lane # 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIG. 18A

FIG. 18B